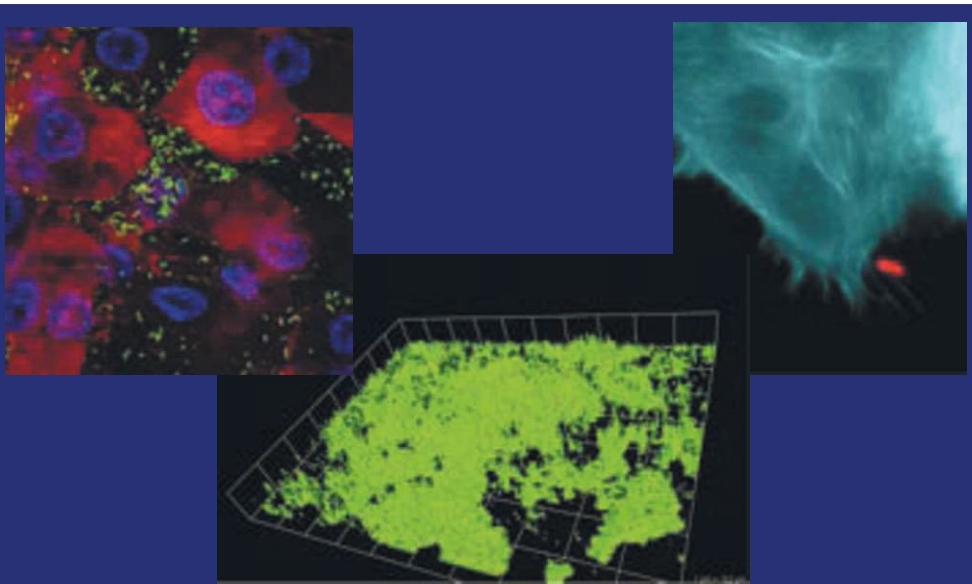
EMERGING INFECTIOUS DISEASES OF THE 21ST CENTURY

MOLECULAR PARADIGMS OF INFECTIOUS DISEASE

A Bacterial Perspective



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Molecular Paradigms of Infectious Disease

A Bacterial Perspective

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MOLECULAR PARADIGMS OF INFECTIOUS DISEASE: A Bacterial Perspective
Edited by Cheryl A. Nickerson and Michael J. Schurr

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Molecular Paradigms of Infectious Disease

A Bacterial Perspective

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Library of Congress Control Number: 2005938810

ISBN-10: 0-387-30917-9 e-ISBN 0-387-32901-3 Printed on acid-free paper.
ISBN-13: 978-0387-30917-0

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Printed in the United States of America. (SPI/EB)

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This book is dedicated to the editors' respective parents, Dr. and Mrs. Max A. Nickerson, and Mr. and Mrs. John C. Schurr, and to Jill Schurr.

Preface

Infectious diseases caused by bacterial pathogens are a leading cause of human death and illness worldwide. In addition to causing significant morbidity and mortality bacterial diseases impose an enormous financial burden on society. Recently, the challenges of effectively treating and preventing bacterial infections have been complicated by (1) the emergence of new organisms and diseases; (2) reemerging strains whose incidence had previously declined; (3) changing patterns of well-known diseases; (4) increased antibiotic resistance in many strains; and (5) the potential misuse of bacteria as agents of bioterrorism. The complexity of these challenges is made even clearer as researchers continue to discover novel mechanisms of bacterial pathogenesis and begin to fully appreciate the diverse ways that bacteria cause disease in humans. Collectively, these issues continue to provide important challenges at both the basic research and clinical levels, and highlight the continued need for an improved understanding of the mechanisms of bacterial pathogenesis.

The study of bacterial pathogenesis has changed dramatically over the last decade, as a result of revolutionary changes in biotechnology and our understanding of molecular and cellular biological systems. This information has greatly enhanced our understanding of bacterial pathogens and how they cause disease. Indeed, in light of the genomic era that has dawned since 1995, it has become increasingly apparent that many bacteria utilize similar methods to become successful pathogens. Therefore, this book is structured to emphasize paradigms of infectious disease that have emerged in the last 10 years.

This book is designed to provide students (both undergraduates and graduates) of the biological and medical sciences with a fundamental understanding of the complex cellular and molecular processes that are important for bacterial virulence and the infectious disease process. In addition, this book serves as a useful text/reference for scientists and researchers of bacterial pathogenesis. Every chapter starts with a boxed section that provides students with a historical overview of critical discoveries that have been accomplished in that specific area of bacterial pathogenesis. A summary

section at the end of each chapter provides a review of the major points of the chapter text. In addition, a question-and-answer section is included at the end of each chapter to help students assess their fundamental understanding of the topic covered.

We have arranged the book in three basic parts. The first highlights key techniques and methodologies that have driven recent discoveries in bacterial pathogenesis including basic genetic and molecular techniques, genomics, and genetic analyses that have been used to identify virulence factors. The second focuses on major structures and mechanisms in bacteria that are important for the pathogenesis/virulence of these organisms. The third concentrates on the regulation of these virulence determinants by global regulators. Since interruption of global regulatory mechanisms abrogates many different bacterial virulence determinants, a better understanding of these pathogenic mechanisms will allow the development of novel therapeutics targeted against a wide range of bacterial pathogens.

In closing, we would like to thank our respective postdoctoral mentors and role models in bacterial pathogenesis, Dr. Roy Curtiss III (C. Nickerson) and Dr. Vojo Deretic (M. Schurr) for their training, support, and generosity. We would also like to thank our respective families and friends for their patience, support, and understanding during the long process of preparation and production of this book. We are especially grateful to Jamie Dominique for her expert assistance in the formatting of this book and her patience in incorporating our many changes into the manuscript.

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Chapter 1

Genetic Analysis of Bacterial Pathogenesis

JAMES M. SLAUCH

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Historical Landmarks

1975 So, Boyer, Betlach, and Falkow clone the gene encoding the heat stabile enterotoxin (ST) from enterotoxigenic *Escherichia coli*, the first example of cloning a "virulence factor" (So et al., 1976).

1976 Casadaban develops a generally applicable technique for constructing transcriptional fusions to the *lac* operon (Casadaban, 1976). These techniques become more facile over the next decade providing powerful methods to study transcriptional and post-transcriptional regulation in bacteria (see Silhavy and Beckwith, 1985; Silhavy et al., 1984).

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1981 Garfinkel and Nester use random transposon Tn5 mutagenesis and screen for avirulent *Agrobacterium tumefaciens* mutants (Garfinkel and Nester, 1980). This is quickly followed by a series of studies where the investigators used transposon mutagenesis and screened for mutations that affect virulence or symbiosis in animals and plants (e.g., Chakravorty et al., 1982; Corbin et al., 1982; Meade et al., 1982; Nida and Cleary, 1983; Normark et al., 1983; Portnoy et al., 1983; Waalwijk and de Graaff, 1983; Weiss et al., 1983; Zimmerman et al., 1983).

1985 Isberg and Falkow identify *Yersinia* Invasin by random cloning into non-invasive *E. coli* and selecting for invasive isolates (Isberg and Falkow, 1985).

1985 Manoil and Beckwith and Hoffman and Wright develop PhoA translational fusions to identify envelope proteins in Gram-negative bacteria (Manoil and Beckwith, 1985; Hoffman and Wright, 1985).

1987 Taylor, Miller, Furlong, and Mekalanos use TnPhoA to identify the toxin coregulated pilus (Tcp) in *Vibrio cholerae* (Taylor et al., 1987).

1987 Osbourn, Barber, and Daniels develop a "promoter-probe" plasmid to identify *in planta* induced genes in *Xanthomonas campestri* (Osbourn et al., 1987).

1988 Falkow puts forth his "Molecular Koch's Postulates" (Falkow, 1988).

1993 Mahan, Slauch, and Mekalanos develop in vivo expression technology (IVET) to identify in vivo induced genes in animal pathogens (Mahan et al., 1993a). This is followed by a series of variations on the IVET theme (see Table 1).

1995 Hensel, Shea, Gleeson, Jones, Dalton, and Holden develop signature tagged mutagenesis (STM), which allows negative selection of bacterial genes required during growth in the host (Hensel et al., 1995).

1995 The first complete genome sequence of a bacterium, *Haemophilus influenzae*, is published (Fleischmann et al., 1995).

1998 Akerley, Rubin, Camilli, Lampe, Robertson, and Mekalanos introduce genomic analysis and mapping by in vitro transposition (GAMBIT) to identify bacterial genes required under a particular condition (Akerley et al., 1998).

2001 Sassetti, Boyd, and Rubin develop transposon site hybridization (TraSH), which uses genomic microarrays and random transposition to identify bacterial gene required under a given condition (Sassetti et al., 2001).

1. Introduction

Free-living organisms often adapt to changes in their environment. These can be relatively simple adjustments that allow the organism to use a new carbon source, or a more complex transformation such as a pathogen adapting to

colonize a particular niche in the host. Indeed, bacterial pathogens must often adapt to multiple host environments in order to survive and propagate—their primary purpose. Pathogens adapt to the host environments by inducing genes that encode virulence factors, which specifically interact with the host to colonize or to circumvent the immune response, as well as metabolic genes appropriate to the particular niche.

Many pathogens are capable of surviving and propagating in a variety of host issues, and often regulate gene expression both temporally and spatially. In order to understand the molecular mechanisms of bacterial pathogenesis, one needs to identify the gene products required for adaptation to these host environments. However, the host is usually a "black box." In other words, we do not understand the host environments, and therefore we cannot mimic these environments in the laboratory in order to ask what genes are regulated in response to, or are required under, these conditions. A primary goal, therefore, in pathogenesis is to identify the genes and gene products that are required for growth and survival in the host. Many investigators have applied the techniques of bacterial genetics that have been so useful in the study of bacteria in the laboratory to bacterial pathogenesis. Select examples will be discussed in this chapter. These techniques were designed for use in various animal models of infection and will be described in this context. However, it should be noted that these techniques simply compare gene expression or gene requirements under two environmental conditions and have applications in areas other than pathogenesis.

2. Fusion-based Techniques for Identification of Virulence Genes

2.1. TnPhoA

Studying the regulation of gene expression is simplified by the use of gene fusions. Rather than monitoring the function of individual proteins (often a difficult task), the regulatory signals of the protein are connected to reporter genes whose expression can easily be assayed (Slauch and Silhavy, 1991). Fusions come in two types. In a transcriptional fusion the reporter gene encodes its own translational start site but is dependent on the promoter of the gene of interest for transcription. In a translational fusion, the open reading frame of the gene of interest is fused with the open reading frame of the reporter gene. The result is a hybrid protein where, for example, the amino terminal portion of the hybrid is encoded by the target gene and the carboxy terminal domain is the reporter. In this case, production of the reporter protein is dependent on the transcription, translation, and, perhaps, localization signals encoded by the gene of interest.

One of the first applications of fusions to the study of bacterial pathogenesis took advantage of the properties of PhoA translational fusions. The *Escherichia coli* PhoA protein is an alkaline phosphatase that is normally

secreted to the periplasmic space. Localization requires recognition of the N-terminal signal sequence by the "sec" secretion system (de Keyzer et al., 2003). The signal sequence is cleaved during export and the mature protein is released into the periplasm. Enzymatic activity of PhoA is dependent on export to the periplasm; the protein will not function in the reducing environment of the cytoplasm (Derman and Beckwith, 1991). Manoil and Beckwith (1985) constructed transposon Tn5 derivatives (see Chapter 2) containing a *phoA* open reading frame lacking the normal signal sequence. The *phoA* is positioned in the transposon such that when the element transposes into a gene in the correct orientation and reading frame, translation of the target gene proceeds through *phoA* producing a hybrid protein (Figure 1). If the target gene is normally localized to an extracytoplasmic location as directed by an N-terminal signal sequence, then the fusion protein will also be exported and the PhoA will be active (Manoil et al., 1990). Thus, one can screen for active PhoA fusions after random transposition into the chromosome and identify extracytoplasmic proteins. Numerous derivatives of TnPhoA have been developed and detailed protocols are available (Manoil, 2000).

Taylor et al. (1987) reasoned that some proteins important for interaction between a pathogen and the host would be localized to the surface of the bacterium. They also reasoned that important virulence factors would be coordinately regulated. They knew that the *ctxAB* operon, which encodes the cholera toxin (CTX), a major virulence factor in *Vibrio cholerae*, is regulated

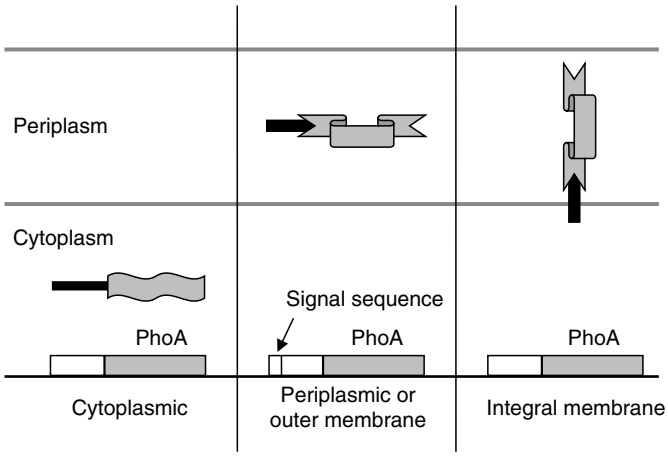

FIGURE 1. PhoA fusions identify envelope proteins. Insertion of TnPhoA in frame with a target protein results in production of a hybrid protein. If the hybrid protein is localized to the cytoplasm, PhoA does not fold appropriately and is nonfunctional. If the fusion protein is targeted to the periplasm via an amino-terminal signal sequence or if the fusion joint corresponds to a periplasmic portion of a polytopic inner membrane protein, PhoA is active.

at the transcriptional level by ToxR in response to certain environmental signals. They randomly transposed the TnPhoA transposon into the chromosome of *V. cholerae* and subsequently screened active PhoA fusions (fusions to exported proteins) for those that were regulated in the same manner as *ctxAB*. They thereby identified the "toxin-coregulated pilus" Tcp. We now know that Tcp is a major virulence factor in *V. cholerae*. The pilus is required for colonization of human intestines and serves as the receptor for the CTX bacteriophage, which encodes the CTX (Davis and Waldor, 2003).

Bacterial two-component regulatory systems (see Chapter 13) are conserved proteins that sense environmental signals and respond, often by changing gene expression. Miller et al. (1989) tested the role of a series of two-component systems in the virulence of *Salmonella typhimurium* and showed that the PhoPQ system was critical in this bacterium. They subsequently screened a series of random PhoA fusions for those that are either induced or repressed when the PhoPQ system was activated. They successfully identified a large number of genes, many of which have subsequently been shown to be important for *Salmonella* to adapt and grow in macrophages. For example, PhoPQ activates genes whose products alter the integrity of the outer membrane and represses genes encoding a type III secretion system (see Chapter 9) that is required at another stage of infection (Groisman, 2001).

Insertion of the TnPhoA into a gene almost certainly disrupts the function of that gene. Thus, one can ask if the gene is important for virulence by testing the insertion mutant in an appropriate infection model. It was shown that many of the PhoPQ-regulated TnPhoA insertions in *Salmonella* conferred a virulence defect in a mouse model (Belden and Miller, 1994; Miller et al., 1989). However, there is a caveat to this experiment. Although the function of the target gene is almost certainly compromised, a fusion protein is being produced. It is possible that any phenotype conferred is due not to loss of function of the target gene, but rather to a toxic effect of the fusion protein. Indeed, subsequent analysis showed that deletion of some of the genes originally identified as attenuating PhoA fusions in *Salmonella* did not confer a virulence defect (Gunn et al., 1995; Miller et al., 1992).

2.2. In Vivo Expression Technology

In vivo expression technology (IVET) takes advantage of transcriptional gene fusions to directly select for bacterial genes that are transcriptionally active during infection. The selected genes are subsequently screened for those that are transcriptionally inactive during growth in laboratory medium, thus identifying genes that are transcriptionally induced when the pathogen is in the host. A subset of those genes that are induced in host tissues include virulence genes that are specifically required for the infection process. Detailed protocols and reviews are available (Angelichio and Camilli, 2002; Slauch and Camilli, 2000).

Various IVET systems have been used to identify in vivo induced genes in both prokaryotic and eukaryotic pathogens (Table 1). These IVET systems are of three types: (1) selection systems based on metabolic (e.g., *purA*) or antibiotic (e.g., *cat*) reporters; (2) recombination-based systems; and (3) green fluorescent protein (GFP) based systems. The IVET selection system was developed in *S. typhimurium* (Mahan et al., 1993a; Slauch and Camilli, 2000).

The original IVET system is based on the fact that mutations in the *purA* gene, encoding adenylosuccinate synthetase, required for synthesis of adenosine 5′-monophosphate (AMP), dramatically attenuate survival of *Salmonella* in host tissues (McFarland and Stocker, 1987). In the animal, this provides a means to select for genes that are transcriptionally active in vivo by creating transcriptional fusions to a promoterless *purA* gene. In order for any given clone to survive and replicate in the host, it must contain a *purA* fusion to a promoter that is transcriptionally active enough to overcome the parental PurA deficiency. The fusion strains that survive in the host are

TABLE 1. IVET systems and selections.[a]

Organism	Selection/Screen	Reference
Xanthomonas campestri	*cat*	Osbourn et al. (1987)
Salmonella typhimurium	*purA-lacZY* (pIVET1)	Mahan et al. (1993a); Slauch et al. (1994)
Vibrio cholerae	γδ resolvase (pIVET5)	Camilli et al. (1994)
Listeria monocytogenes	*lacZ*	Klarsfeld et al. (1994)
S. typhimurium	*cat-lacZY* (pIVET8)	Mahan et al. (1995)
Pseudomonas aeruginosa	*purEK*	Wang et al. (1996a, b)
S. typhimurium	GFP	Valdivia and Falkow (1997); Valdivia and Ramakrishnan (2000)
Yersinia entercolitica	*cat*	Young and Miller (1997)
Escherichia coli	*cat*	Khan and Isaacson (1998)
Staphylococcus aureus	γδ resolvase	Lowe et al. (1998)
Actinobacillus pleuropneumoniae	*luxAB-ribBAH*	Fuller et al. (1999)
Streptococcus gordonii	amylase-*cat*	Kilic et al. (1999)
P. fluorescens	*panB-lacZY*	Rainey (1999)
Candida albicans	Flp recombinase	Staib et al. (1999)
L. monocytogenes	*hly*	Gahan and Hill (2000)
P. putida	*pyrB-lacZ*	Lee and Cooksey (2000)
Histoplasma capsulatum	URA5	Retallack et al. (2000)
Klebsiella pneumoniae	*galU*	Lai et al. (2001)
S. suis	*erm*	Smith et al. (2001)
Shigella flexneri	*cat-lacZ*	Bartoleschi et al. (2002)
P. syringae	*hrcC-uidA*	Boch et al. (2002)
Porphyromonas gingivalis	*tetA-galK*	Wu et al. (2002)
P. fluorescens	*dapB-lacZY*	Gal et al. (2003)
Lactobacillus reuteri	*ermGT-bglM*	Walter et al. (2003)

[a] Listed in chronological order.

subsequently screened for those that are transcriptionally inactive in laboratory medium. This subset of strains contains fusions to genes that are transcriptionally active only during infection. In other words, these genes are specifically induced in the host.

The IVET system was designed with several properties. First, the fusions are constructed in single copy in the chromosome. This avoids any complications that can arise from the use of multicopy plasmids. Second, the fusions are constructed without disruption of any chromosomal genes. If the gene of interest encodes a product required for the infection process, then a fusion that disrupts the gene would not be recovered in the selection. Third, the fusion provides a convenient method to monitor transcriptional activity both in vitro and in vivo.

The pIVET1 plasmid is based on the "suicide vector" pGP704. Replication of pGP704 derivatives requires the replication protein, Pi, the product of the *pir* gene, which must be supplied *in trans*. Therefore, the plasmid will replicate autonomously in Pi^+ strains. In strains lacking Pi, the plasmid must integrate into the chromosome in order to be stably maintained (see below). The plasmid also contains the *cis*-acting site (*mob*) that allows broad host-range conjugal transfer by plasmid RP4 (see Chapter 2).

The pIVET1 plasmid (Figure 2) contains a synthetic operon composed of a promoterless *purA* gene followed by a promoterless *lac* operon, encoding

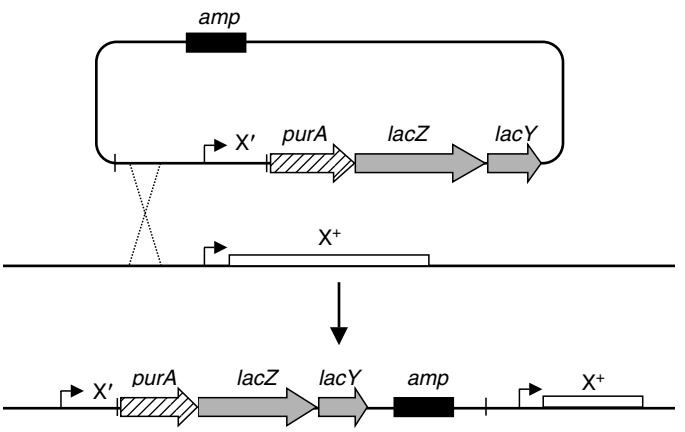

FIGURE 2. Construction of IVET fusions. Random chromosomal fragments are cloned into the IVET vector. Approximately one half of the clones will contain an appropriately positioned promoter that will control expression of the polycistronic *purA lacZ lacY* operon. Introduction of the resulting plasmid into a Pi^- strain where the plasmid cannot replicate and growth in the presence of ampicillin selects for strains in which the plasmid has integrated via homologous recombination. This results in a single-copy fusion controlled by the chromosomal promoter. This strain is also a merodiploid with a wild-type copy of the gene of interest. Note that the background strain is Δ*purA*.

β-galactosidase (LacZ) and Lac permease (LacY). Upstream of the *purA* gene is a unique BglII restriction site. Random *Sau*3AI fragments of *S. typhimurium* chromosomal DNA, isolated from a Δ*purA* strain, are cloned into the *Bgl*II site, 5′ to the *purA* gene in pIVET1. This often results in transcriptional fusions in which *S. typhimurium* promoters drive the expression of a wild-type copy of *purA* and *lacZY*. Conjugal introduction of the pool of fusions into a Δ*purA* strain of *S. typhimurium* that lacks the *pir* gene, and selection for resistance to ampicillin, demands the integration of the plasmids into the chromosome by homologous recombination with the cloned *Salmonella* DNA. In clones that have a promoter in front of *purA*, this integration event results in single-copy diploid fusions in which one promoter drives the expression of the *purA-lac* fusion and the other promoter drives the expression of the wild-type gene (Figure 2).

The integration event is key to the design of the system. The cloned chromosomal sequences provide the only site of homology between the plasmid and the chromosome. The *purA* gene is from *E. coli*, which has diverged sufficiently from *S. typhimurium* to prevent recombination (Rayssiguier et al., 1989). In addition, *S. typhimurium* does not possess a *lac* operon. Note also that only fusion plasmids that contain the promoter from the operon of interest will maintain synthesis of the wild-type gene upon integration (the event drawn in Figure 2). Clones that do not contain a promoter in the correct orientation are unlikely to answer the selection. For example, clones that contain an internal fragment of an operon will generate a fusion under the control of the chromosomal promoter, but will disrupt the expression of the wild-type gene. If the gene encodes a virulence factor, then loss of the gene product could result in selection against this construct in the animal. Likewise, clones that are in the wrong orientation with respect to the fusion will never express *purA* and will, therefore, be selected against in the animal.

In practice, chromosomal DNA is partially digested using the restriction enzyme *Sau3*AI and the fragments are size-fractionated. These fragments are then cloned into the *Bgl*II site in the IVET vector. In the original experiment, fragments of approximately 5 kilobases (kb) were selected (Mahan et al., 1993c). This facilitates integration of the plasmid into the chromosome and increases the likelihood that any clone will contain the promoter region for the gene located at the fusion joint. The number of independent clones should be sufficiently large to ensure a fusion to every gene in the chromosome. How is this determined? The chromosome of *S. typhimurium* is approximately 4.5×10^6 basepairs (bp) and the average gene is about 1,000 bp. The number of independent clones required to ensure, with 95% confidence, that there is a fusion every 500 bp is approximately 54,000. This is calculated from the formula $N = \ln(1-P)/\ln(1-f)$, where P is the probability (0.95 in our example) and f is the "fraction of chromosome" (500 bp/4.5×10^6 bp; Clarke and Carbon, 1976). Because there is an equal probability that any given clone will be in the wrong orientation with respect to the fusion joint, the number is multiplied by 2 in this case. Note that this calculation is

based on the interval between fusion joints (500 bp), not on the size of the clones (5 kb).

The relative activity of the fusions in the population can be monitored by plating the bacteria on lactose indicator medium, e.g., Lactose MacConkey Agar (Figure 3). At least 50 percent of the clones should be phenotypically Lac$^-$, because half of the time the fusion is in the wrong orientation with respect to the gene. This mixed population of fusion strains can now be used to infect an animal model or incubated under other conditions that potentially mimic a host environment. During *Salmonella* infection of a mouse, the requirement for PurA activity is absolute, and only those strains that contain active fusions survive, independent of anatomic location. In other words, the selection is as strong in the intestine as it is during systemic infection. Indeed, the selection has been used to identify genes that are transcriptionally active only in particular tissues of the animal (Stanley et al., 2000).

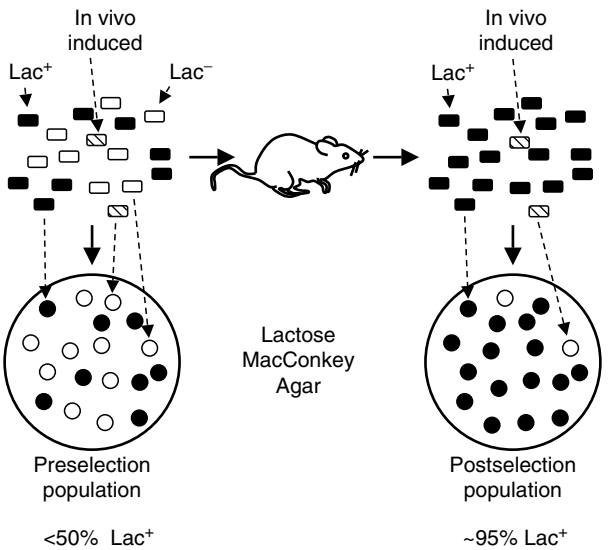

FIGURE 3. The IVET selection. The preselection population of fusion strains includes those where the fusion is highly expressed under laboratory conditions (phenotypically Lac$^+$/Pur$^+$) and those that are not expressed under these conditions (phenotypically Lac$^-$/Pur$^-$) as assayed on lactose indicator medium such as Lactose MacConkey. The latter includes a subset of fusions that will be induced during growth in the host. In order for any given fusion strain to survive passage through the animal, the fusion must be expressed to confer a Pur$^+$ (Lac$^+$) phenotype. The postselection population reflects this, in that the vast majority of strains that survive are those that have highly expressed (Lac$^+$/Pur$^+$) fusions. A small fraction of selected strains are phenotypically Lac$^-$ on laboratory medium. These are fusions to putative in vivo induced genes.

The population of bacteria recovered from any given selection can again be monitored by plating the bacteria on lactose indicator medium (Figure 3). The percentage of colonies that are phenotypically Lac^+ should be dramatically increased in the recovered population; there has been a strong selection for active fusions. The strains of interest, however, are those that are phenotypically Lac^-. These strains presumably survived the selection because the fusion was transcriptionally active in the animal. However, the fusion has low-level expression outside of the animal on laboratory medium. Note that the choice of laboratory medium is somewhat arbitrary. Indeed, the IVET selection simply compares gene expression under two different conditions. Changing those two conditions will alter the repertoire of genes identified. For example, performing the selection in an animal and then monitoring the population on laboratory medium at 37˚C will not identify genes that are temperature-regulated.

The Lac^- strains presumably contain fusions to genes that are transcriptionally induced in the host. To identify these genes, the fusion plasmids are reisolated. The fusions were integrated via homologous recombination (Figure 2). These recombination events are constantly taking place. Hence, in some fraction of a population of cells, the plasmid exists as a separate circular piece of DNA, although it cannot autonomously replicate. These plasmids can be isolated by performing a plasmid preparation starting with a sufficiently large volume of cells. The plasmid is then transformed into a Pi^+ strain in which the plasmid can replicate. Alternatively, because the IVET plasmid has a transfer origin, these plasmids can be recovered by conjugation (see Chapter 2) into a Pi^+ background using a helper plasmid (Rainey et al., 1997). Also, in *Salmonella*, the plasmids can be recovered by P22 bacteriophage-mediated transduction (see Chapter 2) into a Pi^+ background (Mahan et al., 1993b). Once recovered, the gene of interest is identified by DNA sequence analysis using a primer that hybridizes within *purA* oriented to sequence across the fusion joint into the cloned DNA.

There are caveats to the IVET procedure as originally designed. First, the fusions that are identified must have levels of activity that fall within a certain range. The fusion must be phenotypically Lac^- and Pur^- under noninducing conditions. Likewise, the fusion must be induced sufficiently to be phenotypically Pur^+ during the selection. A gene that is transcriptionally induced during infection, but whose basal level of expression outside of the animal is such that a fusion confers a Lac+/Pur+ phenotype will never be identified. A second caveat is amplification of the fusion. Integration of the fusion plasmid by homologous recombination results in a tandem duplication. Recombination between these duplications during chromosome replication can result in multiple copies of the IVET plasmid in the chromosome (Figure 4). These recombination events are constantly taking place and all fusions can amplify. This raises the possibility that a given fusion, which is phenotypically Pur^-, can give rise to Pur^+ cells via amplification rather than transcriptional induction and thereby survive selection in the animal. The same recombination

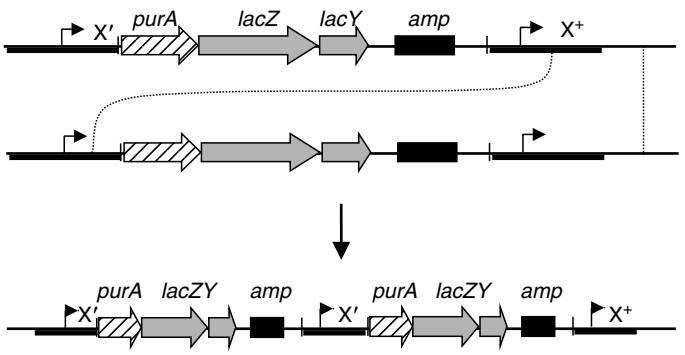

FIGURE 4. Amplification of IVET fusions. Integration of the IVET fusion plasmid generates a "tandem duplication," a stretch of homology (thick lines) corresponding to the original cloned fragment. As the chromosome replicates, recombination between sister chromosomes can result in a duplication of the fusion on one of the chromosomes. In this situation, the amount of transcription of *purA-lac* has doubled, even though expression from any given promoter is unaltered. Note also that the duplication has generated an even larger region of direct homology, and this process can continue to generate further amplification of the fusion. Thus, a fusion with an inherent level of expression that confers a Pur$^-$/Lac$^-$ phenotype can give rise to Pur$^+$/Lac$^+$ cells with no change in promoter activity. Note also that these amplifications are unstable, in that intrachromosomal recombination will resolve the amplification/duplication down to the original single fusion.

events that generate the amplification can resolve the constructs to single copy. This is a false-positive. The strain was phenotypically Pur+ in the animal, but then resolves to give a Lac$^-$/Pur$^-$ phenotype on laboratory medium.

In all cases, one must prove, by independent means, that the identified gene is induced during infection. This can be accomplished by molecular methods such as RT-PCR. Genetically, this is accomplished using a competition assay. Equal numbers of two test strains are mixed and used to infect animals. The infection is allowed to proceed and total bacteria are recovered from whatever site is most pertinent to the experiment or animal model being used. A homogenate of the tissue is plated to determine the total number of bacteria present and then replica-plated to determine the percentage of each strain present in the recovered sample. (The two strains are usually genetically marked with differential antibiotic resistance.) The competitive index (CI) is calculated as (% strain A recovered/% strain B recovered)/(% strain A inoculated/% strain B inoculated). The CI is analyzed statistically using a student's *t*-test.

To test an IVET fusion, one can choose two separate fusion strains from the starting population (preselected) that have levels of expression, as monitored by β-galactosidase activity, that approximate both the uninduced level of the fusion of interest (phenotypically Lac$^-$) and the induced level (Lac$^+$;

Figure 5). If the strain being tested contains a fusion to a gene that is truly in vivo induced, it will compete well when mixed 50:50 with the Lac$^+$/Pur$^+$ control strain and injected into an animal. Likewise, the in vivo induced fusion strain should dramatically outcompete the Lac$^-$/Pur$^-$ strain in a similar assay. If, on the other hand, the selected strain is a false-positive resulting from amplification, it will not compete with the Lac$^+$ control strain, but will behave like the Lac$^-$ strain, because it, like all fusions, can amplify such that a fraction of the cells survive in the animal.

IVET has successfully identified in vivo induced genes in a variety of pathogens (Table 1). Indeed, in *Salmonella*, the technique has identified genes in all of the major virulence regulons except the SPI1 invasion system (our data, not shown, and Heithoff et al., 1997). Note, however, that the technique is designed to simply identify genes that are transcriptionally induced. This often includes metabolic genes, in addition to genes whose products are considered "virulence factors." The definition of "virulence factor" is hotly debated, but many would include the property that a null mutation in a virulence gene would confer a virulence defect. Is this universally true? IVET, as designed, maintains the wild type copy of the gene of interest. Thus, in order to test for a role in virulence, one must specifically create a null mutation in the identified gene and test the resulting mutant in an infection model. Perhaps not surprisingly, some of the genes identified by IVET in a variety of pathogens do not confer a clear virulence defect (Camilli and Mekalanos, 1995; Khan and Isaacson, 1998; Lowe et al., 1998; Merrell and Camilli, 1999; Wu et al., 2002). This does not mean that these genes play no role in the host and the ability to identify

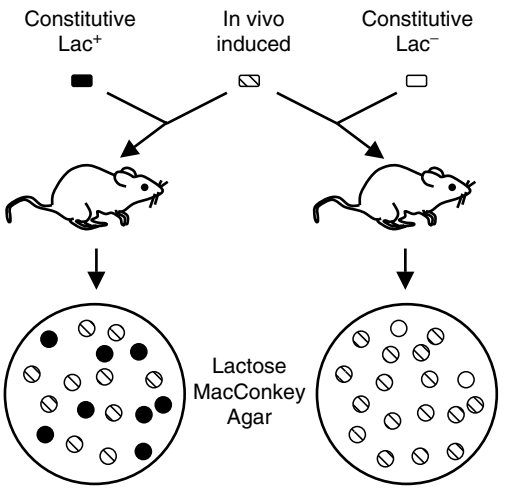

FIGURE 5. Genetic verification of in vivo induced fusions. A competition assay can be used to confirm that a fusion is truly in vivo induced. A constitutive Lac$^+$ fusion strain (always "on") and a constitutive Lac$^-$ fusion strain (always "off") are used as controls. The in vivo induced fusion strain will compete efficiently with the Lac$^+$ strain. (In this figure, the in vivo induced fusion strain colonies are specifically indicated; they are phenotypically Lac$^-$.) The in vivo induced strain will significantly outcompete the constitutive Lac$^-$ strain, although a few of these cells can survive due to amplification.

these genes makes IVET a nice complementary method to those techniques described below that specifically uncover virulence defects.

2.3. Variations on the IVET Theme

IVET has been successfully adapted for use in other pathogens and several variations on a theme have been devised (Table 1). This includes systems based on antibiotic resistance. In these cases, random chromosomal fragments are cloned upstream of a promoterless antibiotic resistance cassette encoding, e.g., chloramphenicol acetyltransferase (CAT). Selection is performed by adding chloramphenicol to an infection model. In theory, one should be able to adjust the level of antibiotic and, hence, the level of expression required to answer the selection, allowing the identification of different classes of genes with different levels of induction. However, the pharmacodynamics of any drug makes this difficult in whole animal experiments. Systems based on fusions to the GFP with screening via fluorescence-activated cell sorting have also been developed (Bongaerts et al., 2002; Valdivia and Falkow, 1997; Valdivia and Ramakrishnan, 2000) but will not be discussed here.

Camilli and colleagues developed a system based not on a selectable marker, but on recombination (Angelichio and Camilli 2002; Camilli et al., 1994; Slauch and Camilli, 2000). In recombinase-based in vivo expression technology (RIVET), transcriptional fusions are constructed as described above, except that the reporter gene is a site-specific recombinase, TnpR, from transposon γδ. When a fusion is induced and the recombinase is produced, it acts on a construct located elsewhere in the chromosome, an antibiotic resistance cassette flanked by recognition sites for the recombinase. The recombinase performs site-specific recombination on the sites removing the resistance cassette from the chromosome. This results in a heritable change; descendents of this bacterial cell are antibiotic-sensitive (Figure 6).

Random chromosomal fragments are cloned into the RIVET construct and integrated into the chromosome by homologous recombination as in Figure 2. The resulting strains are then screened for those that remain antibiotic-resistant and are phenotypically Lac^{-}. This subset of transcriptionally inactive fusion strains is collected by patching the appropriate colonies onto medium with tetracycline (Figure 7). This population is then introduced into the appropriate infection model. The bacteria that are recovered from the animal are screened for those that are antibiotic-sensitive due to recombinational loss of the antibiotic resistance cassette. These strains presumably contain a fusion to a gene that, although transcriptionally inactive in the laboratory, was induced in the host. The fusion can be identified as above and also reintroduced into the antibiotic-resistant parent strain. This rebuilt strain can be used in the infection model and the degree of resolution can be quantified (Angelichio and Camilli, 2002; Slauch and Camilli, 2000).

RIVET has important differences from IVET. First, it is not a selection, but a screen. All of the fusion strains are fully virulent and should compete

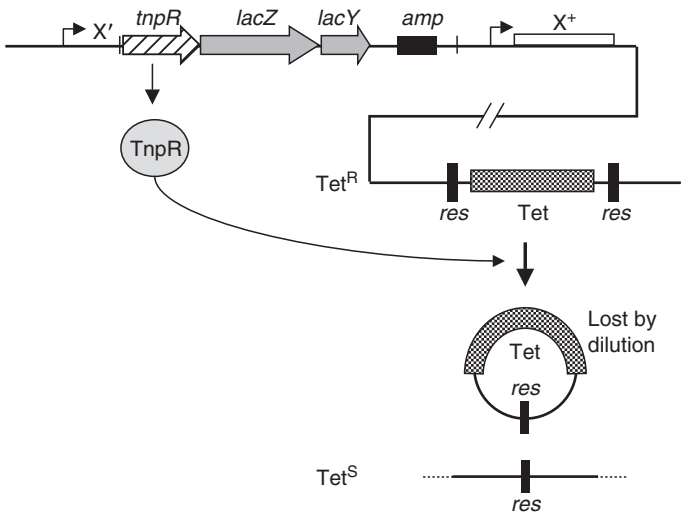

FIGURE 6. RIVET fusions. RIVET fusions are constructed as in Figure 2, except that the reporter gene encodes TnpR, γδ Resolvase. When expressed, this site-specific recombinase acts on a construct located elsewhere in the chromosome. Site-specific recombination between tandem "res" sites results in loss of the tetracycline resistance cassette. The small circular DNA that is created by this event cannot replicate and is lost by dilution as cells replicate. The background strain is also resistant to another antibiotic, e.g., Streptomycin (Sm), to allow unbiased recovery from animal tissues that include endogenous normal flora.

equally. This also means that there is no selection for amplification. Second, the recombinase-mediated loss of the antibiotic resistance marker is a heritable change. This means that, in theory, a fusion that is induced in a limited number of cells, e.g., in a specific anatomic location, could be identified. This property also means that genes that are transiently induced during infection could be identified. In contrast, the IVET system presumably requires that the gene be induced consistently throughout the selection. RIVET has been used both to identify novel virulence factors and to study the temporal expression of these genes during infection (Angelichio and Camilli, 2002). This latter property is a particularly powerful attribute of RIVET that can be applied to genes identified by other methods.

3. Transposon-based Techniques for Identification of Virulence Genes

Genetic analysis often begins with the isolation of mutations that interfere with the biological process of interest, which in pathogenic bacteria is growth and survival in a host organism. Because an avirulent mutant often does not survive the assay, a large-scale random isolation of mutations that affect

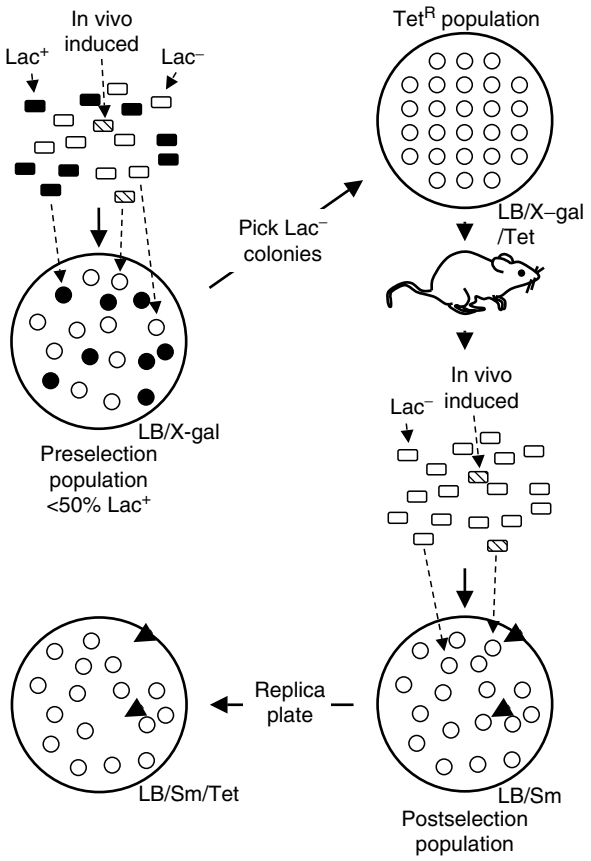

FIGURE 7. The RIVET screen. The preselection population of fusion strains includes those where the fusion is highly expressed under laboratory conditions (phenotypically Lac^+ and Tet^S, due to recombination of the *res*-Tet-*res* construct) and those that are not expressed under these conditions (phenotypically Lac^-/Tet^R). The latter includes a subset of fusions that will be induced during growth in the host. Note, however, that the Lac^- colonies are light blue on the sensitive chromogenic substrate X-gal. The Lac^- colonies are "patched" onto rich medium (Luria-Bertani medium; LB) containing tetracycline to ensure that the starting population has not resolved the *res*-Tet-*res* construct. This population is passaged through the animal and the resulting population is recovered onto LB containing streptomycin (Sm; the background strain is Sm^R). These cells are then replica-plated onto LB/Sm/Tet. Colonies from cells containing fusions to putative in vivo induced genes will be Tet^S (arrows), because at some time during infection, the fusion was expressed, resulting in loss of the Tet resistance cassette.

virulence is difficult. One could screen individual mutants, but this can be very expensive and time-consuming depending on the host. Several more elegant procedures have been developed to identify null mutations in genes whose products are required for the infection process.

3.1. Signature-tagged Mutagenesis

The first procedure is termed signature-tagged mutagenesis (STM; Figure 8; Chiang et al., 1999; Hensel et al., 1995). Detailed protocols are available (Holden and Hensel, 1998). Individual transposons are marked with unique oligonucleotide sequences. In the latest version of STM, 96 mini-Tn5Km2

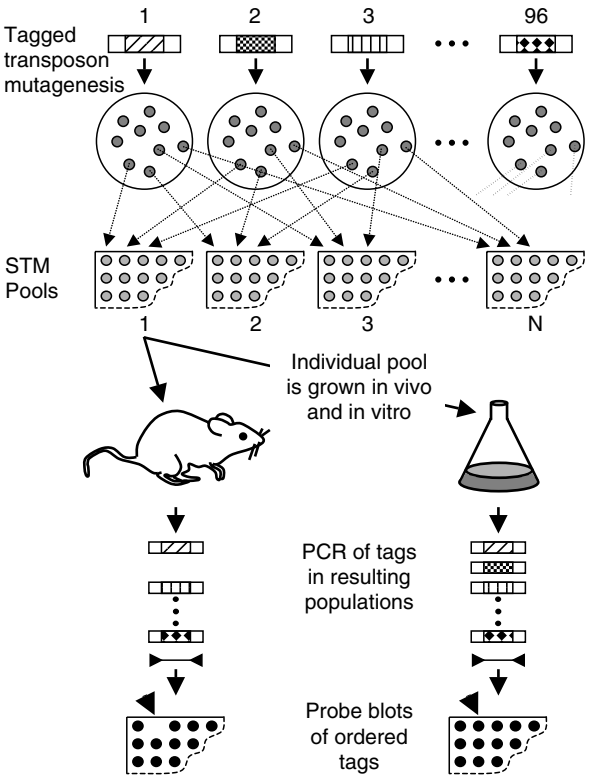

FIGURE 8. STM. A series of 96 transposons (Tn), each containing a unique "tag" sequence (40 bp), are used to mutagenize the pathogen of interest. Pools of 96 tagged mutants are then created by choosing 1 mutant generated with each transposon: 1 colony from the Tn1 mutagenized set is inoculated into well 1 of a 96 well plate; 1 colony from the Tn2 mutagenized set is inoculated into well 2; etc. This procedure generates a series (dependent on the number of 96 well plates) of pools, each of which contains 96 independent mutants that are individually tagged. Each pool is then used to inoculate both laboratory medium and an animal. The resulting populations are recovered and a PCR reaction is carried out that will amplify the tags. The PCR products from the in vitro grown population and the in vivo grown population are independently used to probe a blot, which consists of 96 ordered spots of DNA corresponding to the 96 tags. In the example shown, the mutant in pool 1 generated with Tn2 did not survive in the animal (arrow). This mutant can be recovered from the original 96 well plate and studied further. The transposon in this mutant has presumably inserted into a gene whose product is required during infection.

transposons, each containing a unique 40 bp tag sequence, are used to make random insertion pools. These 96 tags have been predetermined to perform well in the STM procedure (Mei et al., 1997). Each transposon is used to make a pool of insertion mutations in the bacterium of interest. One insertion mutant from each of the individual transposon pools is inoculated into a 96 well plate. This can be repeated until a sufficient number of insertion mutants have been isolated. Each microtiter plate thus contains 96 independent insertion mutants, each of which contains a transposon marked by a unique tag.

The mutants from a given plate are pooled and this population is both grown in laboratory medium and used to inoculate whatever animal model is appropriate. Presumably all the insertion mutants that can grow in the animal will be equally represented at the end of the incubation period. In contrast, those mutants containing insertions in genes that are required during the infection process will be significantly underrepresented in the population of bacteria recovered from the animal. The transposon is designed such that all the unique tags can be amplified by a polymerase chain reaction (PCR). The resulting PCR products from both the laboratory-grown bacteria and the bacteria recovered from the animal are used as probes and are independently hybridized onto a spotted grid containing DNA corresponding to each of the 96 tags. The tag DNA corresponding to the mutants of interest will be hybridized by probes amplified from the laboratory-grown culture, but the appropriate probe will be absent in the reaction amplified from the animal-derived bacteria. Thus, the mutants of interest are defined as missing in the bacteria recovered from the animal. The mutants can be recovered from the original 96 well plate.

There are limitations to this procedure. Depending on the nature of the animal model (or other selection of interest), there may be a limit on the number of mutants that can be coinfected. As the number increases, the probability that a given mutant is lost in the population simply due to random fluctuations increases. Of course the system is designed to use a maximum of 96 mutants at any given time. However, in some systems this number may have to be reduced to 48, 24, etc. Nonetheless, screening these pools of mutants for insertions that affect virulence is far superior to screening individual mutants.

3.2. Genomic Analysis and Mapping by In Vitro Transposition

A second procedure termed "genomic analysis and mapping by in vitro transposition" or GAMBIT (Akerley et al., 1998) was developed and used in *Haemophilus influenzae* and *Streptococcus pneumoniae* (see also Reich et al., 1999). GAMBIT depends on knowledge of the DNA sequence and takes advantage of the natural transformability of these organisms (see Chapter 2). In this procedure (Figure 9), a region of chromosome is amplified by PCR and mutagenized by random transposon insertions generated by in vitro transposition. This mutagenized DNA is transformed and is, in these naturally

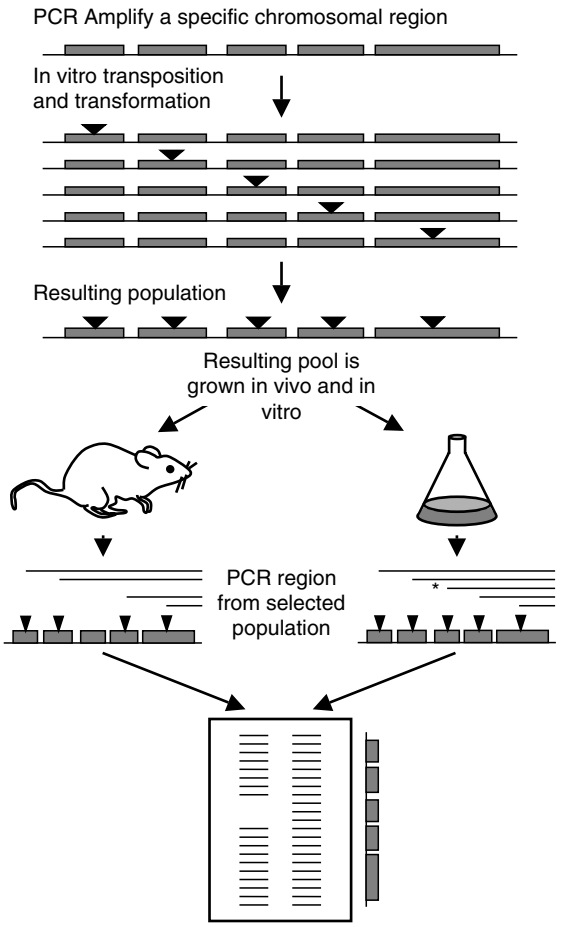

FIGURE 9. GAMBIT. A specific region of chromosome is amplified via PCR. The product is then mutagenized via in vitro transposition. Although any given piece of DNA has only one insertion, the population of fragments contains many insertions in each gene. (Only one insertion is shown for clarity.) Thus, the population can be represented as a chromosomal fragment with multiple insertions (center panel). The mutagenized fragments are transformed and recombine into the chromosome. The resulting population is used to inoculate both laboratory medium and an animal. The resulting populations are recovered and a PCR is carried out. One of the PCR primers corresponds to one end of the chromosomal fragment that was originally amplified, while the other PCR primer hybridizes to either end of the transposon. The length of the resulting PCR product corresponds to the position of each transposon insertion relative to the end of the fragment. In the example shown, mutants containing insertions in the middle open reading frame in this chromosomal fragment did not survive in the animal. Thus, there are no PCR products present in the reaction corresponding to these insertions. These PCR products are recovered from the population grown in vitro (*). The PCR products are directly compared after separation by agarose gel electrophoresis. The "missing" insertions are evident. Note that the mutant is not actually recovered, but because the genome sequence is known, the pertinent open reading frame can be identified and analyzed further. This gene product is presumably required during infection.

transformable organisms, efficiently recombined into the chromosome. Thus, a section of the chromosome is specifically mutagenized. The only assumption is that every gene has an equal probability of being mutated as long as it is not essential for growth in the laboratory. The resulting collection of mutants is used to infect an animal model and bacteria are recovered after sufficient incubation. Analysis of the population is via PCR using one primer at the end of the chromosomal segment that was originally amplified and the other primer that hybridizes to the ends of the transposon. Analysis of both the starting population and the selected population indicates that insertions in a particular open reading frame can or cannot be recovered from the assay (Figure 9). If insertions are not recovered, it is presumed that the encoded gene product is required under the selective conditions. This is analogous to STM in that only a limited number of insertions are used in any given pool. In this case, this is defined by the region of the chromosome that was originally amplified and mutagenized. One needs to perform this procedure multiple times in order to screen the entire chromosome. In contrast to STM, the mutant is not recovered. Rather, once the open reading frame is known, a mutation must be constructed and tested to confirm the role in pathogenesis (see below).

3.3. Transposon Site Hybridization

As designed, GAMBIT is practically limited to those organisms for which the genome sequence is known and which are naturally transformable. Rubin and colleagues (Sassetti et al., 2001; Sassetti et al., 2003; Sassetti and Rubin, 2003) have significantly expanded this concept to any organism where the genome sequence is known and genomic chips are available (a growing list of pathogens; see Chapter 3) by developing the transposon site hybridization (TraSH) method to identify genes required under a chosen condition. TraSH combines the strengths of STM and GAMBIT. It is a powerful method and is discussed in more detail.

In TraSH (Figure 10), the bacterium of interest is randomly mutagenized with a Mariner-based transposon developed for this purpose (Lampe et al., 1999; Rubin et al., 1999; Sassetti et al., 2001). The Mariner transposon is found naturally in insects and is notable because transposition of the element requires only the transposon-encoded transposase but no host factors. Thus, Mariner-based transposons can be used in any organism. The TraSH Mariner has two important properties. First, the transposase is provided *in trans*. It acts on the ends of the element to mediate transposition, but once transposed the element is stable. Second, there are T7 promoters at both ends of the element such that, in the presence of T7 RNA polymerase, transcription will initiate and extend out of the element into adjacent DNA. The transposon is delivered into the bacterium of interest, usually on a nonreplicating piece of DNA such as a defective bacteriophage or nonreplicating plasmid. Transposase, encoded on the same piece of DNA, but separate from the element per se, should act in any organism to mediate transposition from the nonreplicating DNA to the chromosome.

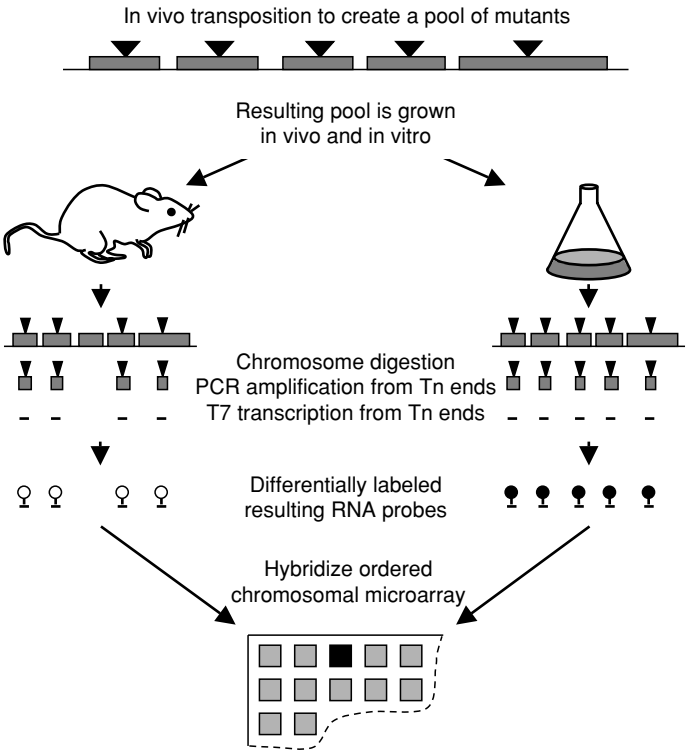

FIGURE 10. TraSH. The pathogen of interest is mutagenized with the MarT7 transposon. Although each mutant cell contains only a single transposon insertion, the population of mutants can be represented as a chromosome with multiple insertions in each gene. (Only a portion of the chromosome with only one insertion in each open reading frame is shown for clarity.) The mutagenized population is used to inoculate both laboratory medium and an animal. The resulting populations are recovered; total chromosomal DNA is isolated and digested with a restriction enzyme that cuts the chromosomal DNA often but does not cut within the transposon. DNA corresponding to the ends of the transposon and the adjacent chromosomal DNA is amplified via PCR. The product is then used as a template for in vitro T7 RNA polymerase transcription initiated from T7 promoters at the ends of the transposon. This overall procedure generates RNA probes that correspond to the chromosomal sequences immediately adjacent to the transposon insertions present in each population. The probes from the two resulting populations are differentially labeled and hybridized to an ordered microarray of DNA representing open reading frames from the pathogen of interest. In the example shown, mutants containing insertions in the middle open reading frame of this portion of chromosome did not survive in the animal. Thus, only the probe generated from the in vitro grown population hybridizes to the spot corresponding to this open reading frame. Note that the mutant is not actually recovered, but because the genome sequence is known, the pertinent open reading frame can be identified and analyzed further. This gene product is presumably required during infection.

Integration will confer antibiotic resistance and hence the mutants can be selected from the original population.

A pool of random insertion mutants should include cells with insertions in all genes that are not essential under the in vitro conditions. This population of mutants or subsets of these can be used to infect an animal model. The bacteria are recovered at the end of the infection. As in GAMBIT, mutants containing insertions in genes required during the infection should be significantly underrepresented in the bacteria recovered from the animal. These genes are identified by comparing the population of bacteria recovered from the animal to a population from the same inoculum grown in laboratory medium. In TraSH, this is done by probing a microarray representing the bacterial genome. The probes consist of sequences immediately adjacent to the chromosomal insertions (see Figure 10).

The method of synthesizing probes from the chromosomal DNA of the recovered bacteria starts with chromosomal digestion and a relatively complicated PCR procedure designed to specifically amplify the ends of the transposons with 250–500 bp of adjoining chromosomal DNA. This is followed by T7 polymerase-mediated transcription of these amplified fragments (Sassetti et al., 2001). This combination provides both signal amplification and specificity. The resulting RNA (after DNase treatment) from each population (grown in vitro versus in vivo) is labeled with a dye and the two probes are used to probe the arrays.

This method identifies insertion mutations that were present in the starting population, but are absent in the population grown in the animal. These insertions are presumably in genes whose products were required in the animal. Although the population of insertions was random, the specific gene of interest is known because each of the microarray spots represents a known gene. A specific mutation in the gene of interest needs to be isolated and tested to confirm the role of the gene in pathogenesis (see below).

The caveats for TraSH are the same as for STM. Specifically, this is a competition assay and the number of different mutants that can be effectively screened in a given inoculum must be empirically determined. For STM, the system is designed such that the maximum number of insertions that is tested in any given inoculum is 96. For TraSH, the number could potentially be much higher. Let us take *Salmonella* as an example to help us think through the issues.

The chromosome of *S. typhimurium* is approximately 4.5×10^6 bp and the average gene is about 1,000 bp. Calculated as above, the number of independent transposon insertions required to ensure, with 95% confidence, that there is an insertion every 500 bp is approximately 27,000. It is relatively straightforward to obtain this number of independent mutants, but should one simply pool all of the mutants and infect animals? The potential fate of a given mutant must be accounted for. The oral LD_{50} for *Salmonella* in Balb/C mice is approximately 10^5. If one orally infects mice with 10^7 mutants, for example, the 27,000 insertions are well represented in the population.

However, the vast majority of bacteria in the inoculum will not colonize the animal, but will rather be excreted in the feces. Are each of the 27,000 mutants well represented in those bacteria that have colonized the intestine and are replicating? This is less clear. Thus, many of the insertion mutants could be underrepresented in the final population not because they have insertions in genes required for colonization or growth in the animal, but because they never colonized in the first place.

The opposite problem can also occur. The LD_{50} of *Salmonella* for infection of BALB/c mice by the intraperitoneal route is <10 bacteria. In order to represent our entire population of mutants, one would have to inject at least 10^5 bacteria; 10^6 or 10^7 would be better. However, at this level of inoculum, the infection will proceed rapidly. It is possible that an insertion mutation that confers a subtle defect in virulence will not have enough time or generations to be selected against in the population. The mutant would still be present in the final population. With TraSH, the probe is produced using PCR and T7 transcription. Therefore, the answer is basically "Yes" or "No"; the insertion is either present or not. Thus, a slight underrepresentation of a mutant may not be evident.

The number of mutants that can be used in a competition assay at one time must be empirically determined for each organism, infection model, route of infection, and dose. The simplest method is to genetically mark a strain in such a way that virulence is unaffected and to mix the marked strain with wild type at various ratios: 1:10, 1:100, 1:1,000, etc. These mixtures are then tested under the same conditions that will be used in the ultimate selection. The largest ratio that routinely returns the marked strain at approximately the same ratio as in the inoculum represents the largest number of insertions that can be reliably used in a single infection. Note that if that number is relatively small, e.g., 1,000, then one would be better off screening a number of smaller pools of independent mutants than screening the larger pool many times. In our example, one would make 27 pools of 1,000 insertion mutants and screen each once, rather than screening the pool of 27,000 mutants 27 times. Of course, the in vivo selected population must always be compared with an in vitro grown population started with the identical inoculum, not just a representative mixture of the starting pool. This ensures that the two populations being compared are, at least initially, identical.

4. Classic Bacterial Genetics in an Animal Model

The techniques described above are all designed to identify genes whose products are important for growth or survival under a particular condition, like in an animal. Often the initial identification of a gene involved in a process is the easy part. Subsequent proof that the gene product plays an important role and analysis of its specific function are the challenge. Even though the functional assays used to determine a role in virulence can be

complex, e.g., infection of whole animals, classic bacterial genetics can contribute significantly to this analysis, and certain genetic requirements must be fulfilled if one is not going to be misled. First and foremost, one must prove that the mutation identified in any of the screens above actually causes the virulence defect. This requires either the movement of the mutation into an otherwise wild-type background or the creation of an independent null mutation in an otherwise wild-type background and subsequent testing of the new mutants. Additionally, one can complement the defect by introducing a copy of a wild-type allele into the mutant and showing that virulence has been restored. In some organisms, these are not trivial experiments, but their importance cannot be overemphasized.

More sophisticated genetic analysis can also provide important information on the role of gene products in virulence. There are several accepted methods for testing the effects of mutations on the virulence of an organism. These include classic tests such as determining the LD_{50} (Reed and Muench, 1938; Welkos and O'Brien, 1994), and also time to death assays, in which small groups of animals are infected with either wild-type or mutant strains and the number of animals that succumb to infection is monitored over time. An attenuating mutation either delays or abrogates lethality.

The virulence assay that is most amendable to genetic analysis is a competition assay (see above), which is highly reproducible, sensitive over a wide range of attenuation, and provides a very powerful system for the analysis of genetic networks in the animal, specifically to determine the relationship between genes whose products are involved in virulence. Beuzon and Holden (2001) have also emphasized the power and utility of this general approach. Are two gene products (1) in the same pathway, (2) affecting the same process via different pathways, or (3) unrelated to one another, independently affecting survival in the host? Genetic analysis can distinguish these models, and examples of each relationship are given below.

The given examples all center on resistance to phagocytic superoxide in *Salmonella*. Macrophages and neutrophils produce a series of reactive oxygen species resulting from the production of superoxide by the NADPH-dependent oxidase (Clark, 1999). Bacteria also produce superoxide in their cytoplasm by the inadvertent transfer of an electron to oxygen via the electron transport chain (Messner and Imlay, 1999). *Salmonella* counteracts these sources of superoxide by producing several superoxide dismutases (SODs), both in the cytoplasm (SodA and SodB) and in the periplasm (SodCI and SodCII). The periplasmic SodCI is encoded on a fully functional bacteriophage termed Gifsy-2, which is lysogenized into virulent strains of *S. typhimurium*. In a recent analysis of the contribution of the Gifsy-2 bacteriophage to *Salmonella* virulence (Ho et al., 2002), it was shown that the phage encodes two major virulence genes: *sodCI* and *gtgE*. Strains containing mutations in *sodCI* have a CI of 0.15 (Table 2). In other words, they are approximately sevenfold decreased in virulence compared to the wild type. Likewise, mutations in *gtgE* confer a sevenfold decrease in virulence. The remaining

TABLE 2. Genetic analysis using competition assays.[a]

Strain A	Strain B	Median CI[b]	No of mice[c]	*P*[d]
sodCI	WT	0.15	9	<0.0005
Δ*gtgE*	WT	0.14	5	0.0006
ΔGifsy-2	WT	0.0068	4	<0.0005
grvA	WT	3.8	25	<0.0005
grvA sodCI	*sodCI*	0.76	15	NS
Δ(G-2B)[e]	WT	0.11	7	<0.0005
grvA Δ(G-2B)	Δ(G-2B)	6.7	3	0.023
Δ*sodA*	WT	1.1	6	NS
Δ*sodB*	WT	0.12	5	0.007
Δ*sodA* Δ*sodB*	Δ*sodB*	0.003	4	<0.005

[a] Data from Ho and Slauch (2001), Ho et al. (2002), and Craig and Slauch (unpublished).
[b] Competitive Index = [output (Strain A/Strain B)/ inoculum (Strain A/Strain B)].
[c] Competition assays are performed i.p. in BALB/c mice.
[d] Student's *t*-test is used to compare the output and the inoculum. NS = not significant.
[e] The Δ(G-2B) deletion removes *gtgE*.

genes on the phage, several of which are known to be involved in host interaction, combined to give a minor threefold effect. Are these gene products related to one another? We concluded that all of these genes act independently based on the fact that the attenuation in virulence caused by deleting the entire phage (~150-fold) is simply the combination of loss of *sodCI*, *gtgE*, and the remaining phage genes ($7 \times 7 \times 3$).

An additional gene carried on the Gifsy-2 phage is *grvA* (Ho and Slauch, 2001). Mutations in *grvA* confer the unusual phenotype of increasing virulence in a competition assay; the *grvA* mutant is fourfold more virulent (Table 2). What is the relationship between *grvA* and other genes on Gifsy-2? The results show that *sodCI* is epistatic to *grvA*. The most sensitive test of this is a competition assay between a *grvA sodCI* double mutant and a *sodCI* single mutant. Both strains are attenuated due to the *sodCI* mutation, but that does not affect the competition assay. What is the *grvA* phenotype in a *sodCI* background? The answer (Table 2) is that these two strains are equally virulent. In other words, in a *sodCI* background, the *grvA* phenotype is no longer evident. In contrast, in a *gtgE* background [Δ(G-2B)], loss of *grvA* still confers an increase in virulence, even though the *gtgE* strain is attenuated to approximately the same degree as a *sodCI* mutant and both defects are the result of mutations in the Gifsy-2 phage. Thus, the inability of *grvA* to exert a phenotype in the *sodCI* background is apparently specific. This means that the *grvA* phenotype requires SodCI, suggesting that GrvA normally functions through SodCI.

In the final example, we take advantage of the genetic concept of "synthetic phenotypes." If two gene products contribute independently to the same process, the combination of the two mutations should give a phenotype that is more severe than what would be predicted by the simple addition of the two

individual mutations. This is exemplified by the phenotypes conferred by loss of the cytoplasmic SODs, SodA and SodB. The *sodA* mutant is fully virulent as previously reported (Tsolis et al., 1995). The *sodB* mutant is attenuated fourfold (Table 2). Thus, in the animal, SodB is apparently the predominant cytoplasmic SOD. What happens if we knock out *sodA* in a *sodB* background? A *sodA sodB* double mutant is 380-fold attenuated when competed against the *sodB* single mutant. In other words, *sodA* has no phenotype in a wild-type background but confers a 380-fold defect in a *sodB* background. This synthetic phenotype is expected and provides proof of principle for this genetic approach. Without prior knowledge of the biochemical function of SodA and SodB, we would conclude that they participate in the same cellular process.

5. Conclusions

Genetics provides a powerful set of tools for analysis of complex biological phenomenon. Over the past few decades, genetic tools have been developed specifically to identify genes in bacterial pathogens that are either transcriptionally induced or required for growth in the eukaryotic host. IVET and RIVET use gene fusion technology to select or screen for genes that are transcriptionally induced during infection. Many, but not all, of these genes are subsequently shown to be required for infection. Other techniques, such as STM, GAMBIT, and TraSH, are "negative selections" that identify genes important for pathogenesis by the fact that transposon insertions in these genes are *not* present in a population selected in a host. These techniques continue to evolve, taking advantage of complete genome sequences and microarrays. The expanding technologies have also allowed analysis of organisms that have historically been genetically intractable. This includes the development of in vitro transposition or transposons that will work in any organism. These technologies also have broad versatility. Indeed, the techniques developed for use in animals are really only sampling gene expression or requirements in two different conditions, and these tools can be, and are being, applied to other situations such as environmental microbiology.

Questions to Consider

1. Draw the integrated IVET plasmid in a situation where the cloned fragment is such that the gene adjacent to the *purA* is transcribed in the "wrong" direction. Would this fusion ever be expressed? What if the cloned fragment contains an internal portion of a gene, i.e., both the 5′ and 3′ are missing?

See Figure 11: Approximately 50% of random clones will have the fragment inserted such that the chromosomal gene located next to the fusion joint is in the wrong orientation to promote transcription. The result of integration is shown. The wild-type gene is intact, but the fusion will never be expressed. If a given

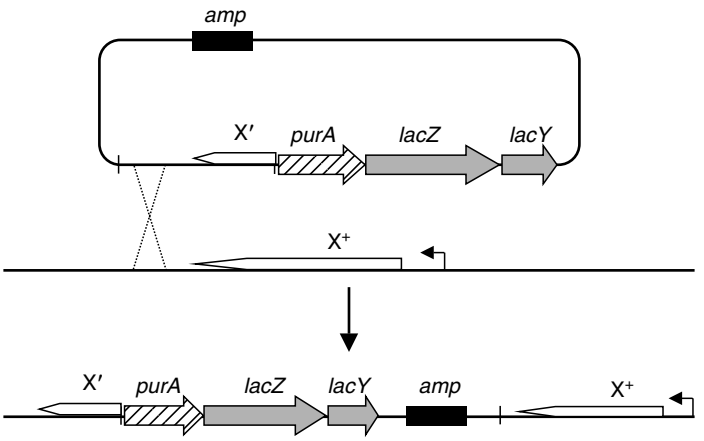

FIGURE 11. Integration of an IVET plasmid in which the cloned fragment is in the wrong orientation with respect to the *purA-lac* fusion.

clone contains an internal fragment of a gene, the wild type copy is destroyed. (This is a common method for gene disruption.) If the fragment is in the correct orientation with respect to the fusion, the fusion will be regulated appropriately. If it is in the wrong orientation, the fusion will never be expressed.

2. In the amplification event drawn in Figure 4, one copy of the chromosome has a duplicated fusion. What happens to the other chromosome?

It has lost the fusion all together. The progeny that inherit this chromosome are Pur^-/Lac^- and will be selected against in the animal.

3. Starting with an amplified IVET fusion (Figure 4), draw the recombination event that resolves the construct back to a single copy.

See Figure 12: Recombination between direct repeats results in a deletion of the intervening sequences; in this case, loss of the plasmid. Since the plasmid cannot replicate, it is lost by diffusion. Note that in a situation where there are multiple copies in the amplified construct one or multiple copies can be lost. The frequency of these events is strictly dependent on the length of the homology. Therefore, all of the events (recombinational loss or amplification) are equally likely. This is a very dynamic situation.

4. Design an experiment to use RIVET to identify genes that are induced only when a pathogen is forming a biofilm (see Chapter 10).

Starting with a random population of fusions, select those that remain tetracycline-resistant during growth in liquid medium. Then allow this population to form a biofilm. Scrape the cells from the biofilms and screen for those that are tetracycline-sensitive. These strains contain fusions to genes that were transcriptionally inactive in liquid, but were induced in the biofilm.

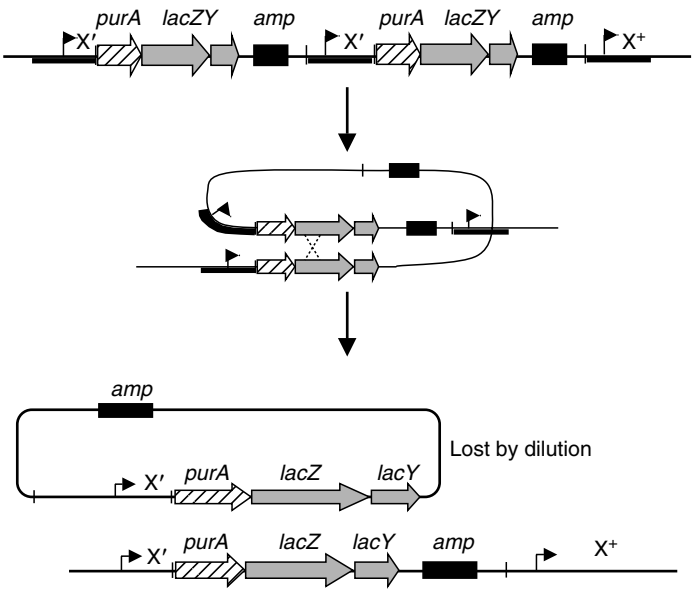

FIGURE 12. Intra-chromosomal recombination between tandemly duplicated fusion plasmids can result in resolution to a single copy.

5. Using STM, you have isolated a mutant that clearly does not survive in your animal model. However, when you move the transposon into an otherwise wild-type background, the attenuated phenotype is lost. What is the explanation?

It is most likely that there are two mutations in the strain. The transposon insertion confers resistance to kanamycin but is not in a gene that affects virulence. However, this strain background contains a spontaneous mutation that does attenuate the organism. In other words, the virulence phenotype does not genetically map to the insertion mutation. It is vital to prove that the insertion actually causes the phenotype.

6. In the transposon techniques, why do we compare in vivo grown strains to in vitro grown strains versus just the inoculum?

This relates to the definition of a virulence factor. Many people would argue that a mutation that just makes the cell sick and grow poorly is not very interesting, even though it will probably affect virulence. Therefore, the mutant population is grown not only in the animal but also in laboratory medium. If a particular mutant cannot compete in laboratory medium, the fact that it cannot compete in the animal becomes less meaningful.

7. You want to perform TraSH, but you decide that you are not interested in auxotrophic mutants, many of which would be attenuated. How do you remove these mutants from your analysis?

Plate the starting population on minimal medium. Auxotrophs will not grow, and therefore will not be present in the inoculum used to infect the animal.

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Chapter 2

Genetic Exchange in Bacteria and the Modular Structure of Mobile DNA Elements

JAMES W. WILSON

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Historical Landmarks

1915–1917 Bacteriophages are discovered independently by Frederick W. Twort in Great Britain and Félix d'Hérelle in France (reviewed in Duckworth, 1976).

1928 Griffith shows that avirulent, nonencapsulated pneumococcus can be "transformed" to become virulent and encapsulated by incubating with heat-killed virulent pneumococcus. He hypothesizes that a substance called transforming principle is transferred from the virulent to the avirulent pneumococcus (Griffith, 1928).

1944 Avery, MacLeod, and McCarty demonstrate that DNA is the transforming principle (Avery et al., 1944).

1946 Lederberg and Tatum discover conjugation when they observe that strains of bacteria can exchange genetic markers. Lederberg later coins the term "plasmid" in a review paper on cellular genetics (Lederberg and Tatum, 1946).

1951 Freeman demonstrates that a bacteriophage can transfer virulence traits from pathogenic to nonpathogenic strains of *Corynebacterium diphtheriae* (Freeman, 1951).

1952 Zinder and Lederberg discover generalized transduction of genetic markers using *Salmonella* bacteriophage (Zinder and Lederberg, 1952).

1952 Using bacteriophages labeled with radioactive protein and DNA, Hershey and Chase provide undisputable proof that DNA is the genetic material (Hershey and Chase, 1952).

1959 First R-plasmid discovered when multiple antibiotic resistance is transferred from *Escherichia coli* to *Shigella* (reviewed in Mitsuhashi, 1977).

1967–1971 Pathogenic properties are observed to be transferred at high frequency between strains of *E. coli*, and the concept of the virulence plasmid is established (reviewed in Miller et al., 1994).

1974–1977 The *Agrobacterium tumefaciens* Ti plasmid is discovered and subsequently shown to insert into the host plant genome (reviewed in Zhu et al., 2000).

1980 Plasmid-mediated tissue invasiveness by *Yersinia enterocolitica* is demonstrated by Zink et al., (1980).

1980–1981 The first conjugal transposons are discovered: Tn*916* from *Enterococcus faecalis* and Tn*5253* from *Streptococcus pneumoniae* (Buu-Hoi and Horodniceanu, 1980; Franke and Clewell, 1981).

1998 A large chromosomal pathogenicity island is shown to be transferred from one bacterial strain to another by a helper bacteriophage (Lindsay et al., 1998).

1. Introduction

Evolution demands that genetic traits be passed on to other members of a given population. The transmission of genetic information in bacteria can be divided into two main modes: vertical and horizontal. Vertical transfer of genes occurs when the bacterial chromosome replicates and each daughter cell receives a chromosomal copy upon cell division, analogous to mitosis and cell division in eukaryotic cells. By contrast, the horizontal transfer of genes occurs between individual members of a population via mechanisms that are not based on chromosomal replication and cell division. Horizontal transmission can be thought of as the bacterial form of sexual genetic transfer and involves mechanisms such as transformation, plasmid conjugation, and bacteriophage transduction. Horizontal transfer allows a genetically unrelated bacterial strain or species to enter a population and transmit its genes into the other cells that are present. In other words, horizontal transfer allows diverse types of bacteria to exchange genes and therefore represents a powerful force to drive evolution.

The mechanisms that have evolved to allow bacteria to transfer genetic information between cells via horizontal transfer are true marvels of nature. The evidence that these genetic exchange mechanisms have played major roles in the evolution and dissemination of virulence genes is vast and very difficult to dispute. The following observations serve as testament to the role of horizontal genetic transfer in the evolution of pathogens:

- A large number of virulence genes are contained on mobile genetic elements such as plasmids, bacteriophages, and DNA regions that are substrates for recombinases that allow their excision from a particular genome. For a number of pathogenic species, such as *Vibrio cholera*, *Staphylococcus aureus*, pathogenic *Escherichia coli*, and others, the presence of a single virulence function on a mobile DNA element is the essential factor for a particular disease.
- There are several examples of large (10–50+ kb) contiguous blocks of chromosomal virulence genes that function together for a specific process, typically for the secretion of virulence factors. Through genomic sequence analysis, it is clear that these regions have been acquired by horizontal

transfer and are absent from genomes of related, nonpathogenic strains or species. These regions have been termed "pathogenicity islands." Frequently, pathogenicity islands are associated with integrases and other genes that are related to mobile DNA elements like plasmids and bacteriophages.

- Large blocks of *related* virulence genes (5–25 genes), such as type III and type IV secretion systems, have been found in the genomes of many different pathogenic species (frequently as part of pathogenicity islands). The genes encoding these systems from different species are clearly homologous and ancestrally related. For such large regions of related genes to have all evolved independently in these different species is highly unlikely. Instead, these genes were most likely disseminated over the course of evolution via horizontal transfer mechanisms.
- The emergence of antibiotic resistance in response to the widespread use of antibiotics worldwide is one of the most impressive examples of the power of microbes to adapt to selective pressure. Analysis of the spread of antibiotic resistance genes among different bacterial species has revealed a central role for mobile DNA elements such as plasmids and transposons in this process.

Put simply, horizontal transfer of genetic information allows bacteria to evolve in ways that vertical transmission of random mutations from mother to daughter cells will not facilitate. Therefore, the study of these genetic exchange mechanisms will reveal a set of strategies that bacteria use to evolve and adapt, frequently in ways that allow them to interact with eukaryotic hosts and cause disease. Table 1 lists numerous examples of bacterial toxins that are contained on mobile genetic elements or large sections of horizontally transferred DNA (Novick, 2003).

The main objective of this chapter is to describe the major genetic exchange mechanisms in bacteria and how different parts of these mechanisms can be utilized in various combinations to create different genetic elements. In addition to learning the basic conceptual themes and mechanisms of genetic exchange, it is becoming increasingly important to be able to combine different parts of the basic concepts in novel ways in order to explain the functioning of several newly discovered genetic elements. As an increasing number of bacterial genomes become sequenced and new genetic elements get discovered, it is apparent that the lines between plasmid, bacteriophage, and transposon are becoming blurred when describing these new elements.

The field of bacterial horizontal gene transfer is vast and includes many concepts that have been shaped by data obtained from highly talented scientists worldwide. Some of the references for individual sections of this chapter are reviews that contain much of the presented information for that section. Additional, specific references (if not provided here) can be found within those reviews.

TABLE 1. Examples of bacterial toxins that are contained on mobile genetic elements.[a]

Disease	Toxin	Organism	Element	Features
Diphtheria	Diphtheria toxin	*Corynebacterium diphtheriae*	β-Prophage	~35 kb
Anthrax	Anthrax toxin	*Bacillus anthracis*	Plasmid	~110 kb
Cholera	Cholera toxin	*Vibrio cholerae*	Prophage	Filamentous
Dysentery	Shiga toxin	*Shigella dysenteriae*	Prophage	Lambdoid
Botulism	Botulin toxin	*Clostridium botuliman*	Prophage	110–165 kb
Enterocolitis	Cpe toxin	*Clostridium perfringens*	Transposon	$IS1470_2$::cpe ~6 kb
Gangrene	α-toxin	*Clostridium perfringens*	Chromosome[b]	
Tetanus	Tetanus toxin	*Clostridium tetani*	Plasmid	"Large"
Enterocolitis	Enterotoxin	*Clostridium difficile*	Path Islet	~3 kb
Diarrhea	Labile toxin (LT)	*Escherichia coli*	Plasmid	90 kb F-like
Diarrhea	Stable toxin (ST)	*Escherichia coli*	Transposon	$IS1_2$::cat ~3 kb
Toxic shock syndrome	TSST-1	*Staphylococcus aureus*	Path island	~15 kb ϕ-related
Food poisoning, TSS	Enterotoxin A	*Staphylococcus aureus*	Prophage	~45 kb
Food poisoning, TSS	Enterotoxin B, C	*Staphylococcus aureus*	Path island	~15 kb ϕ-related
Food poisoning, TSS	Enterotoxin D	*Staphylococcus aureus*	Plasmid	~30 kb
Scalded skin syndrome	Exfoliatin A	*Staphylococcus aureus*	Prophage	~45 kb
Scalded skin syndrome	Exfoliatin B	*Staphylococcus aureus*	Plasmid	~30 kb
Necrotizing pneumonia	Leukocidin	*Staphylococcus aureus*	Prophage	~45 kb
Scarlet fever	SPEA, C	*Streptococcus pyogenes*	Prophage	~45 kb

[a] Data from Novik (2003). Reprinted with permission from Elsevier.
[b] *C. perfringens* α-toxin is the single exception to the rule that toxinosis-causing toxins are encoded by mobile genetic elements.

1.1. Vehicles that Mediate Horizontal Gene Transfer

Horizontal transfer is driven by DNA elements that are mobile; they are able to spread from one bacterium to another and can serve as vehicles that can carry large sections of genes between hosts. Certain mobile DNA elements, such as transposons, can "jump" from one DNA element to another; this type of genetic transfer is very important because it allows a section of DNA that is located on a vertically transferred element (a chromsome) to be moved to a horizontally transferred element (like a bacteriophage or plasmid). The transposed DNA section can then be potentially transferred to a variety of other different strains or species. Below is a list of mobile DNA elements that we will refer to in this chapter:

- *Plasmids*—DNA elements that autonomously replicate apart from the host chromosome.
- *Bacteriophages*—viruses that infect bacterial cells using a protein package that contains genetic information, usually DNA though some RNA phages do exist.
- *Transposons*—sections of DNA located between repeated sequences that can be excised and moved to a separate DNA element via a recombination enzyme called a transposase.

It is helpful to think of these genetic elements as combinations of genetic modules or cassettes. Genetic modules contain genes and/or DNA sites that allow mobile DNA elements to move between hosts, jump between genomes, and be stably maintained in the population. Various combinations of certain genetic modules that we will discuss in this chapter give rise to different mobile DNA elements and different mechanisms for their propagation and transfer. However, the descriptions of different mobile DNA elements are based on the three main vehicles: plasmids, bacteriophages, and transposons.

1.2. The Four Major Horizontal Transfer Paradigms: Transformation, Conjugation, Transduction, and Transposition

The study of bacterial genetic exchange has revealed three processes that allow horizontal gene transfer between cells to occur and a fourth process that allows vertically transferred DNA to jump onto horizontally transferred elements and vice versa. *Transformation* is the uptake of naked DNA that has been released into the environment. As part of their life cycles, it is common for bacterial cells within a population to lyse and release their DNA, and bacteria have evolved mechanisms to bind this DNA and import it into their cytoplasm. *Conjugation* is the directed transfer of DNA through a membrane pore from a donor cell to a recipient cell. A single strand of DNA is transferred and is copied in the recipient, while the other single strand is copied in the donor. This process is commonly mediated by plasmid systems that can either mediate their own transmission ("self-transmissible") or transfer other plasmids that express the appropriate functions (termed "mobilizable plasmids"). *Transduction* is genetic exchange that is mediated by bacteriophages. Bacteriophages package certain DNA molecules present in a host bacterium in a protein sheath termed a capsid and can then transfer this DNA to another bacterium upon release of the phage particles from the host. *Transposition* is the movement of a section of DNA from one location in a particular genome, such as a chromosome or plasmid, to another separate genome. This is a critical concept in horizontal gene transfer because it provides an important way for vertically and horizontally transferred genetic elements to exchange genes.

In the following sections, we will discuss the concepts outlined below:

(1) Transformation mechanisms
(2) Plasmid replication, conjugation, and maintenance
(3) Bacteriophages and transduction
(4) Transposons and the transposition of DNA
(5) The modular structure of mobile genetic elements
(6) A world of genetic modules

2. Transformation Mechanisms

Both Gram-positive and Gram-negative bacteria have evolved mechanisms to allow the uptake of naked DNA from the extracellular environment (Chen and Dubnau, 2004). In these systems, the extracellular DNA is recognized and bound by receptor proteins on the outer surface. Another complex of membrane-associated proteins then mediates the physical transport of the DNA across the cell envelope. During this transport, one strand of the DNA is degraded and a single-stranded DNA molecule is introduced into the recipient cytoplasm. The newly transported, single-stranded DNA molecule can then serve as a substrate for the host recombination machinery, which integrates the DNA piece into the recipient's genome via recombination mechanisms. Several of the proteins in both Gram-positive and Gram-negative bacteria that are involved in the DNA uptake process belong to a family of proteins also involved in other processes such as type IV pilus formation, type II and III secretion, and twitching motility (the pilus/secretion/twitching/competence or PSTC family). In the well-studied transformation systems, there is a strong correlation between the expression of type IV–related pilus proteins and the ability to import DNA from the environment.

2.1. Gram-positive Transformation

Most of the information on Gram-positive transformation is derived from experiments with *Bacillus subtilis* and *Streptococcus pneumoniae* (Chen and Dubnau, 2004). Figure 1 illustrates a model of Gram-positive DNA uptake based on the *B. subtilis* system, but the two systems are very similiar. The model can be broken down into two major parts: binding and transport.

- *Binding*—the ComEA protein is a surface receptor that binds the DNA non-specifically and delivers the DNA to the machinery that transports it across the membrane. ComEA is homologous to the C-terminal domain of a kinesin-like DNA-binding protein called Kid. In addition, there is a flexible peptide sequence adjacent to the DNA-binding domain of ComEA that may allow the protein to bend. It is thought that after ComEA binds the DNA, it conformationally changes to hand off the DNA to the

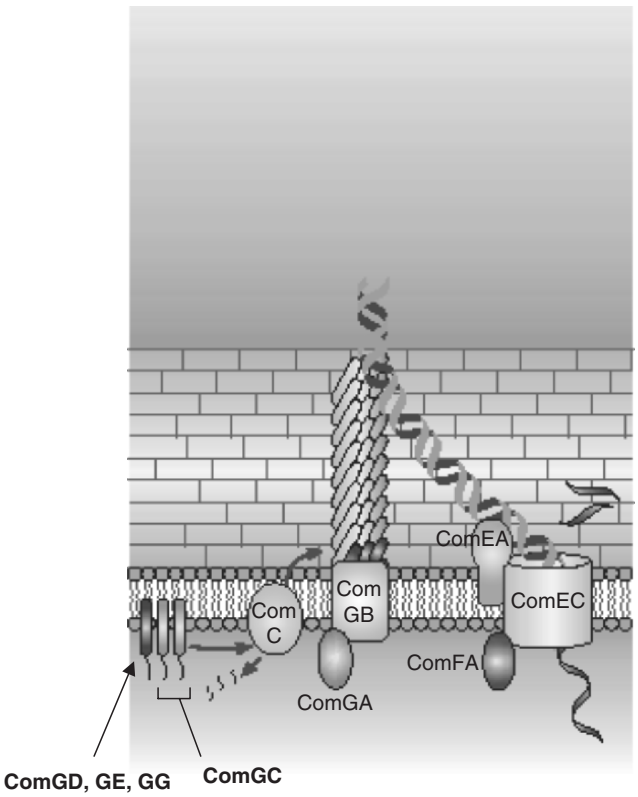

FIGURE 1. Model of DNA uptake during natural transformation of Gram-positive bacteria. On the surface of Gram-positive cells expressing the Com DNA uptake system, ComGB and ComGA aid in the formation of a pilus-like structure consisting of the major subunit (ComGC) and minor subunits (ComGD, -GE, and -GG). The pilus-like structure extends from the ComGB protein in the diagram. These subunits are processed by the ComC prepilin peptidase. This "pseudopilus" is thought to allow the DNA to access the membrane-bound receptor, ComEA, which delivers the DNA to the ComEC channel in the cytoplasmic membrane. The ATP-binding protein ComFA aids in the process of single-stranded DNA transport across the membrane. This model is based on the nomenclature of the *Bacillus subtilis* DNA uptake system. (From Chen and Dubnau, 2004.)

membrane DNA transporter. The ComG proteins (ComGC, ComGD, ComGE, and ComGG), which belong to a class of pilus assembly proteins, form a structure in the outer peptidoglycan layer that provides access of the DNA to ComEA. These pilus-like proteins are processed by the prepilin peptidase ComC. The ComGA and ComGB proteins also aid in the process of forming the ComG pilus-like structure on the cell surface.

- *Transport*—the ComEC protein is a polytopic membrane protein, and ComFA is a membrane-associated protein, which is a member of the DEAD

helicase family (named for its conserved sequence of Asp-Glu-Ala-Asp amino acids), and contains a consensus nucleotide-binding motif. These two proteins together form a transport complex that moves the DNA across the membrane. In addition, a nuclease (termed NucA in *B. subtilis* and EndA in *S. pneumoniae*) serves to degrade one of the DNA strands during transport, possibly providing energy for the ComEC/ComFA-mediated process.

2.2. Gram-negative Transformation

The model for Gram-negative DNA uptake is derived from the *Neisseria gonorrheae* and *Haemophilus influenzae* systems, and can be divided into the same parts as the Gram-positive model above (Chen and Dubnau, 2004). This model uses the nomenclature from *N. gonorrheae* (Figure 2), but the mechanism can be applied to the *H. influenzae* (and other) systems.

- *Binding*—in both the *N. gonorrheae* and *H. influenzae* systems, there are specific uptake sequences (USs) on the naked DNA that are recognized by a receptor on the outer membrane. This is a major difference from the Gram-positive model. For *N. gonorrheae*, the US is GCCGTCTGGA; in *H. influenzae*, it is AAGTGCGGT. Each of these sequences is found at a significantly greater-than-random frequency in each of the respective genomes, indicating that these bacteria preferentially import DNA from their own species. In *H. influenzae*, the DNA is bound at specific locations on the cell surface called "transformasomes," which form a bleb-like subcellular structure in which the DNA is sequestered until transport.
- *Transport*—the following components are involved with DNA uptake in *N. gonnorheae*: (1) PilQ, an outer membrane protein belonging to the secretin family; (2) PilC, a pilus-associated protein; (3) PilE, a pilin-like protein thought to provide a channel through the cell wall like the *B. subtilis* ComG proteins above; (4) ComA, a polytopic inner-membrane protein, which is an ortholog of the *B. subtilis* ComEA protein; (5) Tpc and ComL, which are predicted murein hydrolases; (6) PilF, a traffic NTPase involved in pilus formation; (7) ComP, a prepilin-like protein; and (8) ComE, a periplasmic protein similar to *B. subtilis* ComEA. Together, these proteins allow transport of the naked DNA from the outer surface across the outer membrane, cell wall, and inner membrane to the cytoplasm. There is also thought to be a nuclease, similar to the Gram-positive model, that degrades one of the DNA strands during transport.

2.3. *The* Helicobacter pylori *DNA Uptake Mechanism Is Related to Type IV Secretion*

Helicobacter pylori is a Gram-negative gastric pathogen that uses a transformation mechanism with components related to type IV secretion systems, most notably the plant pathogen *Agrobacterium tumefaciens* T-DNA export system

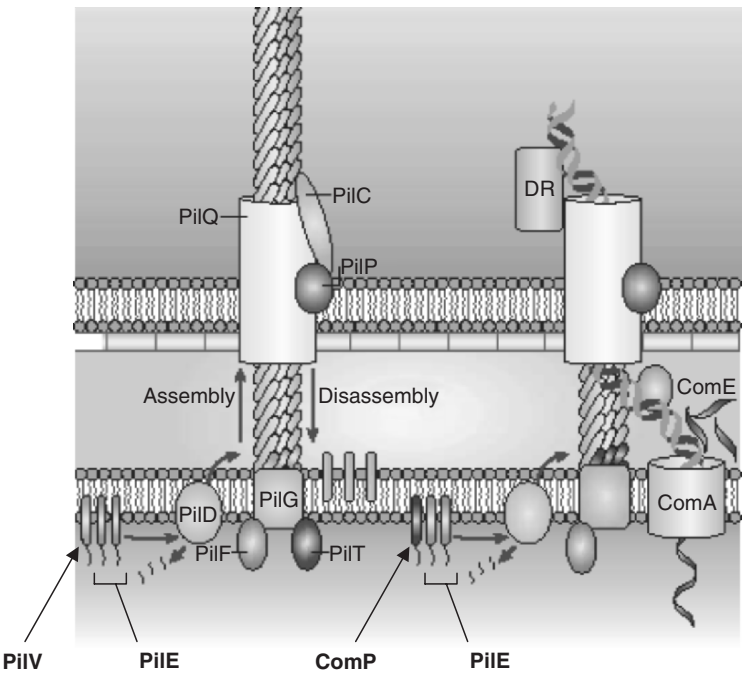

FIGURE 2. A model for DNA uptake during natural transformation of Gram-negative bacteria. This model of DNA uptake in Gram-negative bacteria is based on the system found in *Neisseria gonorrheae*. In *N. gonorrheae*, a type IV pilus is formed on the surface using the Pil proteins. The Pil proteins used to form this surface pilus also function with other Com proteins to facilitate DNA uptake. The left side of this diagram depicts the apparatus involved with type IV pilus formation. The prepilin peptidase (PilD) processes the major (PilE) and minor (PilV) subunits for assembly into the polymerized structure by the PilG membrane protein and the PilF and PilT traffic NTPases. The pilus-like structure extends from the PilG protein in the diagram. The outer membrane secretin protein PilQ, aided by the proteins PilC and PilP, allows the pilus structure to cross the outer membrane. This pilus is able to extend and retract by alternating between assembly and disassembly modes.

The right side of the diagram depicts the model of DNA uptake that involves the Pil and Com proteins. The PilE protein, together with a separate minor pilus subunit (ComP), forms a pseudopilus via the same mechanism as for type IV pilus assembly. This pseudopilus is thought to extend across the periplasm to the PilQ secretin. An as-yet-to-be-indentified DNA receptor (DR) located at the surface binds the DNA and shuttles it into the secretin pore in the outer membrane. The DNA is recognized in the periplasm by the ComE protein and is delivered to a DNA channel in the plasma membrane formed by the ComA protein. A single strand of the DNA enters the cytosol, while the other strand is degraded. (From Chen and Dubnau, 2004.)

(Chen and Dubnau, 2004). In *A. tumefaciens*, the VirB7, B8, B9, and B10 proteins are encoded by colinear genes as part of the T-DNA system, and these proteins are major components of the transmembrane pore through which the T-DNA is transported. In *H. pylori*, four genes of the DNA uptake system, *com*B7–B10, are similiarly arranged, and the protein products (ComB7–B10) resemble their *A. tumefaciens* counterparts. The ComB7–B10 proteins most likely form a membrane apparatus in a manner similar to the *A. tumefaciens* (and other type IV) system(s). ComB7 is an outer membrane protein that is likely bound to ComB9 by a disulfide bond. ComB8, ComB9, and ComB10 form a membrane complex that spans from the inner membrane to the periplasmic space and binds to ComB7. ComH, a protein predicted to be exported to the cell surface (and not a type IV system homologue), is a candidate to be a DNA-binding protein that serves to recognize the DNA substrate and provide nuclease activity that is involved in the processing of the DNA for transport (Chen and Dubnau, 2004).

2.4. Competence Induction

In certain bacterial species, transformation can only take place after the cells in the population have been made "competent" for the uptake of DNA—a process termed "competence induction." Competence induction is an example of a quorum-sensing mechanism: when the cells of the population get to a certain density, a signal molecule is produced in sufficient quantities to induce a regulatory cascade in the cells that changes their physiology. In the case of competence induction, the result of quorum sensing is that the cells express the genes necessary for DNA uptake. One of the best studied competence induction systems is that of *S. pneumoniae*, and this system can serve as an analogous example for other competence systems (Claverys and Havarstein, 2002). In the *S. pneumoniae* system, the competence inducer molecule is expressed from the *comC* gene as a large ComC precursor protein. The ComC precursor is recognized by an ATP-binding cassette (ABC) transport apparatus formed by the membrane-bound ComA and ComB proteins. The ComC precursor is cleaved upon its secretion by ComA and ComB and a small competence-stimulating peptide (CSP) derived from the ComC precursor is exported to the extracellular environment. The CSP is then recognized by other *S. pneumoniae* cells in the population via a surface histidine kinase receptor termed ComD. ComD is a member of a two-component regulatory system with ComE, a transcriptional regulator that gets phosphorylated by ComD upon CSP binding. Phosphorylated ComE then initiates a regulatory cascade that serves to turn on the genes required for DNA uptake. By using competence induction, the cells of a population optimize the control of DNA uptake genes and orchestrate the DNA uptake system to function when conditions are most favorable for DNA exchange.

3. Plasmid Replication, Conjugation, and Maintenance

3.1. Plasmid Replication

The study of plasmid replication has revealed that there are conserved mechanisms used for this process (del Solar et al., 1998; Khan, 1997; Kobryn and Chaconas, 2001; Qin and Cohen, 1998). Most plasmids are covalently closed, circular DNA molecules, but linear plasmid DNA elements do exist as well. These two different molecules have replication mechanisms that are conceptually distinct and are therefore described separately. The major concept of plasmid replication is that of the *replicon* consisting of a cis-acting origin of replication and an initiator protein (most commonly plasmid-encoded) that recognizes the origin and initiates replication. Plasmid replication mechanisms are based on this important scheme. Another important plasmid-related concept is that of *incompatibility*. Plasmids that share common replication and maintenance functions typically cannot coexist in the same bacterial cell and this leads to the loss of one of the plasmids. This is based on the fact that common functions on the plasmids can interact with each other and interfere with normal replication and maintenance of each plasmid. In this case, one plasmid typically "outcompetes" the other and is maintained while the other plasmid is lost. Plasmid replication determinants are common mediators of incompatibility.

The different plasmid replication mechanisms are: (1) theta-type; (2) strand displacement; (3) rolling circle; and (4) linear. We will give brief descriptions of these mechanisms here; however, the reader is directed to several excellent reviews for more details (del Solar et al., 1998; Khan, 1997; Kobryn and Chaconas, 2001; Qin and Cohen, 1998). The overviews of replication presented here serve as conserved models that may be utilized when characterizing the replication modes of newly discovered genetic elements.

3.1.1. Theta-type Replication

For theta-type replication (Figure 3), the origin of replication is bound by the initiator (or "Rep" protein), and the two DNA strands are opened (or "melted") so that two single DNA strands are exposed (del Solar et al., 1998). A DNA polymerase and DNA helicase (these are typically both host-encoded) then start replication at the replication forks that are formed upon melting of the origin. Typically, DNA synthesis is continuous on one strand of the replication fork (and results in the formation of the "leading strand") and discontinous on the other (and results in the formation of "lagging strands"). If only one replication fork is used during DNA synthesis, the replication is termed "unidirectional" and terminates when the replication fork returns to the origin. If both replication forks are used, replication is "bidirectional" and terminates somewhere on the DNA molecule opposite the origin. The term "theta" was used to describe this mode of circular DNA

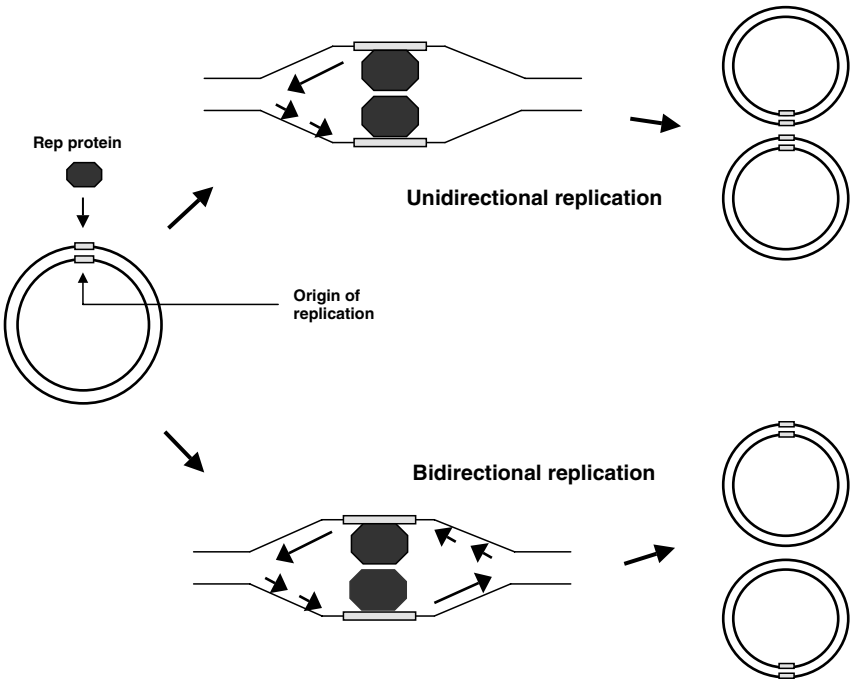

FIGURE 3. Unidirectional and bidirectional theta-type plasmid replication. A circular plasmid is depicted and its origin of replication is indicated. The replication initiator protein (or "Rep protein") recognizes the origin, binds to it, and facilitates origin melting. This leads to host factors being recruited to the origin and commencement of DNA synthesis. Note the formation of leading and lagging strands at the replication forks. In unidirectional replication, only one replication fork is used and replication terminates when the DNA synthesis machinery returns to the origin. In bidirectional replication, both replication forks are used and replication terminates somewhere on the DNA molecule opposite the origin.

replication because when these origins were first visualized via electron microscopy, they were shaped like the Greek character "theta" (θ).

The origin of replication of theta-type plasmids have some common features (del Solar et al., 1998): (1) repeated DNA sequences termed "iterons" that serve as the binding sites for the initiator protein (these plasmids are also described as "iteron-containing" plasmids); (2) A/T-rich sequences that facilitate the opening of DNA origin; and (3) binding sites for host replication factors that aid the replication process, such as DnaA, IHF, FIS, and others. There are some deviations from this scheme, such as plasmid ColE1, which does not use a plasmid-encoded protein initiator but rather a small RNA molecule to prime DNA polymerase I-intitated replication; however, these general features are remarkably conserved among iteron-containing, theta-type plasmids.

3.1.2. Strand Displacement Replication

The strand displacement mechanism of replication is used by the broad-host-range plasmids of the IncQ group (del Solar et al., 1998). It is similar to theta-type replication, but is unique in the following ways: (1) the early stages of replication do not require host-encoded replication factors typically involved in theta-type replication such as RNA polymerase, DnaA, DnaB, DnaC, and DnaG; (2) three plasmid-encoded proteins are utilized for replication: the RepA helicase, the RepB primase, and the RepC origin-binding protein; (3) DNA synthesis is bidirectional, but is exclusively continuous (proceeding as the leading DNA strand is displaced by the RepA helicase). The fact that this mechanism of initiation is independent of host replication factors may help to explain the broad-host-range nature of the IncQ plasmids.

3.1.3. Rolling Circle Replication

The model for rolling circle replication is presented in Figure 4, and it differs significantly from the theta-type and strand displacement mechanisms (del Solar et al., 1998; Khan, 1997). The general mechanistic features of rolling circle replication are as follows: (1) there are two replication origins: a double-stranded origin and a single-stranded origin; (2) the replication initiator protein, in a dimer form for most systems, recognizes the double-stranded origin, nicks a single DNA strand at this site, and becomes covalently attached to this strand at a conserved tyrosine residue; (3) replication is initiated at the exposed 3′-OH DNA group at the nick site via other factors including DNA polymerase III, helicase, and single-stranded DNA-binding protein; (4) termination of this replication cycle results in a newly copied, double-stranded molecule and a displaced, single-stranded molecule; (5) replication of the single-stranded molecule is initiated by host factors at the single-stranded origin and results in a second, newly copied double-stranded molecule.

Rolling circle replication is a highly conserved mode of replication. It is used by single-stranded and double-stranded bacteriophages and many plasmids found in Gram-positive bacteria. In addition, the conjugative transfer of DNA is basically a rolling circle replication mechanism where the displaced single strand is transferred from a donor to a recipient bacterial host.

3.1.4. Linear Replication

The best-known examples of linear plasmids are found in *Streptomyces* spp. and in the Lyme disease agent *Borrelia borgdorferi* (Kobryn and Chaconas, 2001; Qin and Cohen, 1998). The linear *Streptomyces* plasmids, typified by the well-studied pSLA2, have terminal proteins bound to the 5′ ends of the linear molecule, similar to adenovirus or the *B. subtilis* phage ϕ29. In contrast, the *Borrelia* plasmids are covalently closed at each end by an interstrand DNA bond. Both strategies serve to protect the linear plasmids from degradation by exonucleases.

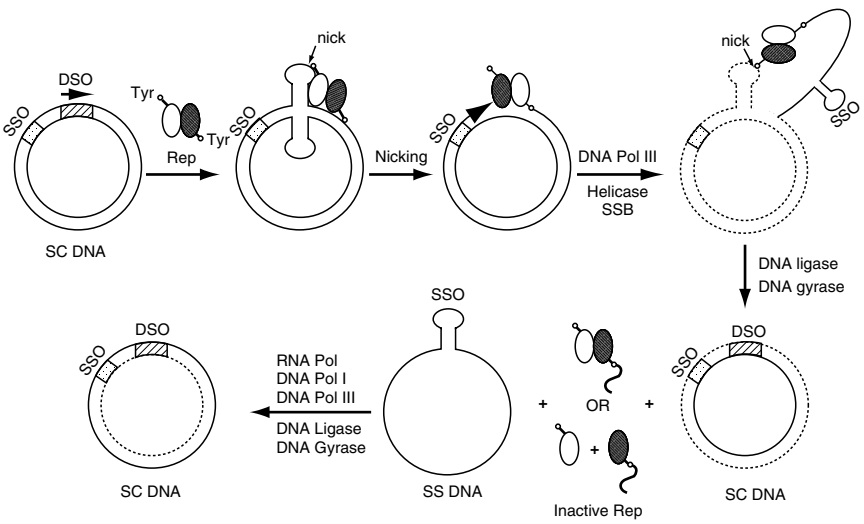

FIGURE 4. Rolling circle replication of a bacterial plasmid. At the double-stranded origin (DSO), the replication initiator protein (Rep protein, shown here as a dimer) forms a nick at a hairpin structure and becomes covalently attached to a single strand of the DNA via a tyrosine residue on one of the monomers. DNA replication is initiated at the exposed 3′-OH DNA group at the nick site via host factors including DNA polymerase III, helicase, and single-stranded DNA-binding protein. Replication of the new strand displaces the strand that is bound by the Rep protein dimer. DSO-mediated replication is terminated when the replication machinery returns to the DSO site, the Rep dimer once again nicks the DSO site (via the tyrosine residue on the other monomer), and the newly replicated strand is ligated together via host DNA ligase. This process also results in the displaced strand being religated (via the Rep protein activity) and the formation of inactive Rep protein molecules, which are bound to small pieces of plasmid DNA that are byproducts of the religation reaction. This DNA byproduct can be initially observed at the nick in the newly replicated DSO where it joins at the tyrosine residue of one of the Rep monomers. The displaced strand is replicated from the single-stranded origin (SSO) via the activity of a number of host factors. This model results in the formation of two double-stranded copies of the plasmid. (From Khan, 1997.)

The replication of *Streptomyces* pSLA2 initiates from an origin located internally within the linear genome and proceeds bidirectionally toward each end. In this model, lagging strand synthesis leads to the formation of a 3′ overhang of between 200 and 300 bp at the ends of the newly replicated DNA molecules. The repair (or "filling-in") of these overhangs is likely to be primed by a "fold-back" mechanism, where inverted repeats at the ends of the genome allow a hairpin structure to form that is recognized by the terminal protein (Figure 5). The terminal protein would aid in the initiation of repair synthesis and become covalently bound to the 5′ end of this new molecule.

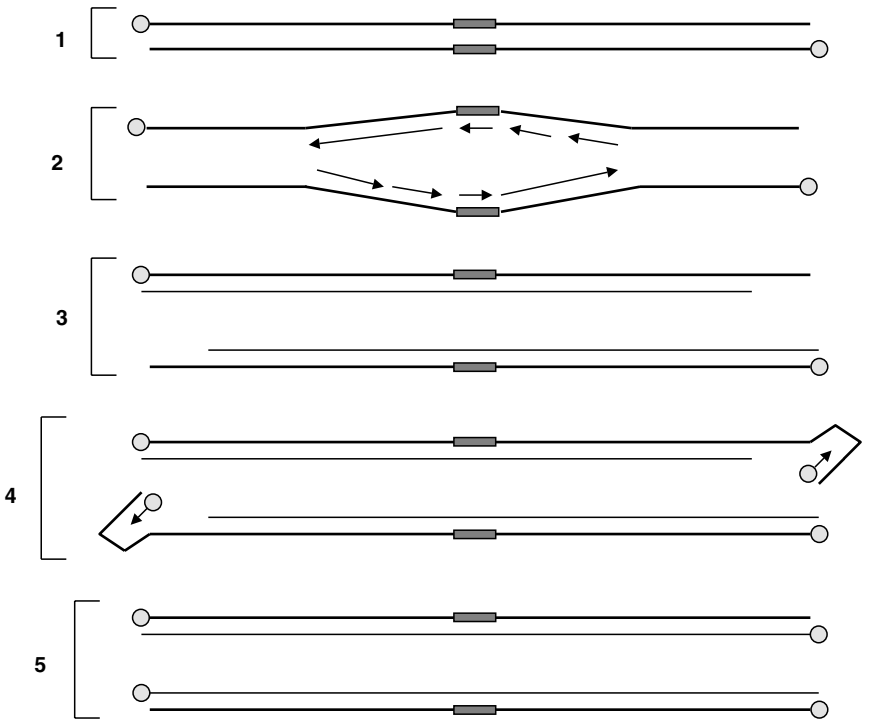

FIGURE 5. Model for replication of linear plasmids that have terminal proteins bound to their 5-primed ends. (1) A linear plasmid with covently bound terminal proteins at each 5′ end is depicted. (2) Bidirectional replication commences at an origin located in the middle of the molecule. Note the formation of leading and lagging strands during DNA synthesis. (3) Lagging strand synthesis results in 3′ overhangs that must be repaired (or "filled in"). (4) A "folded-back" structure forms at the 3′ ends of the overhanging strands. This structure is recognized by the terminal protein, which catalyzes the repair synthesis to fill in the terminal gap. (5) Fill-in repair of the overhangs results in completely replicated molecules that have terminal proteins bound to each 5′ end.

Borrelia plasmids also initiate replication from an internally located origin, but a "head-to-head", "tail-to-tail" dimer replication intermediate is predicted to form (Figure 6). This dimer is resolved into two full linear genomes by a DNA breakage and rejoining reaction catalyzed by the ResT telomere resolvase. This reaction produces the convalently closed telomeric ends of the linear genome.

3.2. Plasmid Conjugation

As described above, conjugation is the replicative, horizontal transfer of DNA from a donor cell to a recipient cell (Cascales and Christie, 2003; Christie, 2001; Llosa et al., 2002). Typically, conjugation involves the

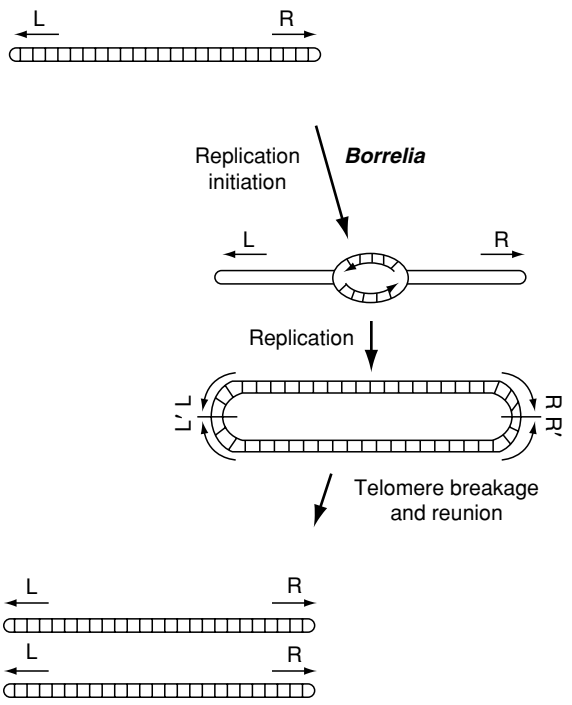

FIGURE 6. Replication of a linear plasmid with covalently closed hairpin ends. A linear plasmid with covalently closed hairpin ends is depicted, and direct repeats present at the left and right telomeric ends of the genome are indicated. This particular example is based on linear plasmids from *Borrelia* spp., but may be applicable to similar linear plasmids that exist in nature. Replication is believed to proceed from internal, bidirectional origins. The replicated telomeres are processed by a DNA cleavage and reunion event that is catalyzed by a telomere resolvase enzyme. (From Kobryn and Chaconas, 2001. Reprinted with permission from Elsevier.)

transfer of plasmid molecules, but the conjugative transfer or mobilization of transposons and bacterial chromosomes also occurs. An important distinction when discussing conjugation is that between self-transmissible and mobilizable elements. Self-transmissible elements encode all the conjugation functions needed to mediate their own transfer from a donor to a recipient (the *oriT*, Dtr, and Mpf components described below). Mobilizable elements are able to conjugatively transfer, but do not encode all the functions necessary for self-transmission. Typically, these elements contain *oriT* and Dtr functions, but not a mating pair formation (Mpf) system. They rely on the Mpf functions from another self-transmissible element for their transfer from donor to recipient.

3.2.1. Conjugation Systems Are Composed of Three Major Components

(1) A cis-acting DNA site termed the origin of transfer or *oriT*;
(2) DNA transfer and replication proteins (Dtr) (also known as DNA-processing functions) that recognize the *oriT* and create a single-stranded nick at this site. The protein that binds the *oriT* and creates the nick is termed the *relaxase* protein. There is also a protein that couples the nicked *oriT*/relaxase complex to the mating pair formation system (see below) termed the *coupling protein*. The Dtr functions are also referred to as "Mob" genes.
(3) Mpf proteins form any pili or fimbrial structures associated with conjugation and also form the conjugal pore that serves as the conduit for DNA transfer. The Mpf components of almost all conjugation systems belong to the type IV secretion family that is described in Chapter 9 on bacterial protein secretion mechanisms. This family of secretion systems has evolved to transport DNA and proteins substrates and has become widely disseminated among bacterial species including pathogens. The Mpf functions are also referred to as "Tra" genes.

3.2.2. Conjugation Is Essentially Rolling Circle Replication Coupled to Type IV Secretion

The mechanism of conjugation is shown in Figure 7. There are four steps in this mechanism, which is very similar to rolling circle replication and type IV secretion (Cascales and Christie, 2003; Christie, 2001; Llosa et al., 2002):

(1) The relaxase protein binds to the *oriT* on the DNA substrate and cleaves one of the DNA strands to create a single-stranded nick. The nicking reaction results in the relaxase becoming covalently attached to the 5′ end of the cleaved DNA strand at a conserved tyrosine residue in the *N*-terminal region of the protein.
(2) The coupling protein, which forms a homo-hexamer and is associated with the Mpf apparatus at the cytoplasmic membrane, facilitates interaction of the *oriT*/relaxase with the conjugation pore. This is how the DNA is "hooked up" to the transfer apparatus.
(3) The coupling protein is able to drive the transfer of the *oriT*/relaxase complex through the Mpf type IV secretion pore and into the recipient cell.
(4) After the initial transfer of the oriT/relaxase complex, the remainder of the single-stranded DNA is pumped through the Mpf pore into the recipient. After this transfer is completed, the ends of the single-stranded molecule are rejoined by the catalytic ligase activity of the relaxase protein, which is released in this reaction.

Conjugation can be thought of as rolling circle replication (where the relaxase plays the role of the initiator protein) that is coupled to type IV secretion (where the coupling protein and Mpf pore are the type IV apparatus). The mechanism described above has been termed the "shoot and pump" model

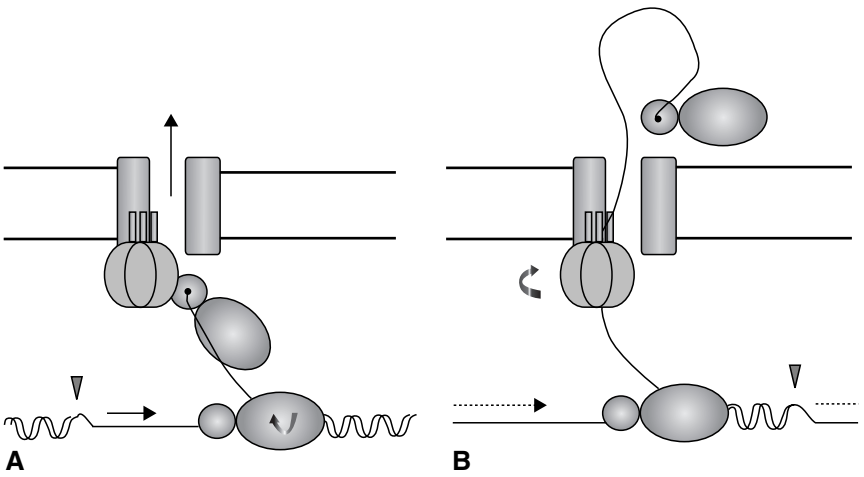

FIGURE 7. A model for DNA transfer during bacterial conjugation. This diagram depicts a conjugative plasmid being processed at its origin of transfer (*oriT*) and its subsequent interaction with the mating pore apparatus that allows transfer of the plasmid to the recipient. The straight, dark lines indicate bacterial envelopes of the donor and recipient (the donor contains the plasmid and is at the bottom of the diagram). A pore is formed between the two cells via the mating pair formation (Mpf) proteins, and the coupling protein is shown to be associated with the mating pore on the donor side. The wavy line indicates the plasmid DNA, and the vertical arrow indicates the location of the single-stranded DNA nick that is produced at the *oriT* via the activity of the relaxase protein (depicted as the two-part oval shapes). The relaxase protein forms a dimer that binds to the oriT region. One subunit of the dimer becomes covalently attached to one strand of the plasmid DNA as a result of the nicking reaction. (A) After the relaxase-catalyzed nicking reaction at *oriT*, the relaxase monomer that is bound to the single-strand of plasmid DNA is recognized by the coupling protein at the mating pore. The relaxase enzyme is then "shot" through the mating pore into the recipient cell via the activity of the coupling protein. Replication of the plasmid DNA in the donor cell proceeds from the *oriT* site (this is indicated by the horizontal arrow) and is aided by the helicase activity of the relaxase monomer that remains associated with the donor plasmid DNA. (B) After shooting the DNA-bound relaxase monomer into the recipient cell, the coupling protein pumps the rest of the single DNA strand into the recipient. When replication of the plasmid DNA in the donor returns to the *oriT*, the relaxase monomer in the donor ligates it together at the nick site and liberates the transferred single strand to complete its transfer to the recipient. Inside the recipient, the relaxase monomer rejoins the transferred strand, and this strand gets replicated into a double-stranded molecule via the activity of a primase (often plasmid-encoded) and host DNA synthesis functions. (From Llosa et al. 2002. Reprinted with permission from Blackwell Publishing.)

because the *oriT*/relaxase complex is initially shot through the Mpf apparatus and the rest of the DNA is pumped into the recipient cell. Conjugation is a beautiful example of two molecular processes (DNA replication and macromolecular secretion) that have been combined to produce a powerful means of genetic exchange.

3.3. Plasmid Maintenance Functions

A plasmid molecule that consists of just a minimal replicon (either with or without a conjugation system) is frequently not maintained efficiently in the cell population and is lost from cells at a high frequency that exceeds the rate of expected random distribution. This could be due to the fact that the plasmid is a metabolic burden on the cells or that the plasmids are not distributed properly upon cell division, among other possible reasons. Therefore, plasmids have evolved mechanisms that ensure their stable maintenance within the bacterial population. The most common way to maintain plasmid vectors in the laboratory is via an antibiotic resistance gene that allows plasmid-containing cells to survive in the presence of a given antibiotic. However, plasmids in nature are often cryptic and do not contain an easily selectable phenotype such as antibiotic resistance. In addition, if a plasmid does contain a resistance marker, the antibiotic is not always present to allow selection for plasmid-containing cells. In these cases, other molecular mechanisms can ensure that all daughter cells inherit a plasmid copy upon cell division. These maintenance mechanisms are extraordinary examples of how extrachromosomal DNA elements have evolved to ensure their persistence within a bacterial population.

3.3.1. Partition Systems

Partition systems, or "Par" systems, serve to actively distribute plasmids to daughter cells upon cell division in a manner that is conceptually similar to centromeres in eukaryotic cell division (Gerdes et al., 2004; Moller-Jensen et al., 2000; Pogliano, 2002). Plasmid molecules are grouped at the midcell division plane, and then copies are moved to areas within each daughter cell so that even distribution is obtained at cell division. This type of distribution has been visualized using plasmid molecules that have been fluorescently tagged, and the distribution has been shown to be dependent on the presence of an active partition system.

The components that form a partition system are remarkably conserved in different bacterial plasmid and chromosomal genomes. A typical partition system consists of the following plasmid-encoded components (with nomenclature based on the well-studied ParAB system of plasmid P1):

(1) A cis-acting plasmid DNA site consisting of several repeated regions (termed ParS in plasmid P1);
(2) A ParA or ParM protein that contains Walker-type or actin-like ATPase domains, respectively;

(3) A ParB protein that binds to the cis-acting site on the plasmid and recruits the ParA or ParM component to this complex.

The ParA or ParM/ParB/ParS complexes that form between copies of plasmid molecules become localized at midcell (possibly via an interaction with a bacterial host cell function) and are thought to be propelled toward the opposite cell poles (or ends) so that cell division compartmentalizes copies of the plasmid in each of the daughter cells. The ATPase activity of the ParA/ParM component is thought to play a critical role in this process. Alternatively, single Par complexes may bind to bacterial host cell functions that are evenly distributed on either side of the midcell but limited in number. There may be just enough of the bacterial host cell functions to bind plasmid copies on either side of midcell to ensure proper plasmid distribution upon cell division. In this way, all the plasmid copies do not bind to one side of the midcell, which would create plasmidless daughter cells.

3.3.2. Multimer Resolution Systems

Frequently, multimers of plasmid copies can form via homologous recombination. This can have the effect of lowering the effective plasmid copy number and decrease the frequency of plasmid inheritance by daughter cells. Plasmids have evolved multimer resolution systems to counteract this phenomenon (Barre and Sherratt, 2002; Sauer, 2002). These systems consist of: (1) a DNA site and (2) a recombinase protein that initiates recombination at the DNA site. Each single plasmid copy contains one DNA site. When two plasmid molecules fuse to form a dimer, there will be two DNA sites contained on this dimer molecule. The recombinase will perform a site-specific recombination reaction between the two DNA sites that will result in the dimer plasmid molecule being resolved back into two separate monomer plasmid copies. The best-known multimer resolution system is the *lox*/Cre system of plasmid P1 and Xer/*cer* of *E. coli* (Barre and Sherratt, 2002; Sauer, 2002). The *lox*/Cre system has been extensively utilized to perform site-specific recombination reactions that allow certain genetic engineering experiments to be performed in a wide array of cell types.

3.3.3. Post-segregational Lethality Systems

Post-segregational lethality systems serve to kill any plasmidless daughter cells that arise in the population (Engelberg-Kulka and Glaser, 1999). These systems are also called "plasmid addiction" or "toxin–antitoxin" systems. A plasmid encoding such a system expresses both a toxin that is lethal to the bacterial cells and an antitoxin that inactivates the toxin. The key point is that the antitoxin component has a very short half-life compared to the toxin component. As long as the plasmid is present in the cell, the antitoxin is produced and the host cell is protected. However, if a plasmidless daughter cell arises, the source of continually expressed antitoxin is gone and any remain-

ing antitoxin degrades very quickly. In this case, the stable toxin is free to mediate killing of the plasmidless host cell.

In the *hok*/*sok* system of plasmid R1, the toxin–antitoxin interaction takes place at the RNA level. The *hok* RNA encodes the toxin protein. The *sok* RNA can hybridize to the *hok* RNA molecule, prevent its translation, and signal for the hybrid to be degraded. Consistent with the model above, the *sok* RNA is quickly degraded in plasmidless cells and translation of the *hok*-encoded toxin commences. In other post-segregational lethality systems (those of F, R100, and RK2), the toxin and antitoxin are proteins that bind to one another in an inactive complex. Degradation of the antitoxin protein in plasmidless host cells releases the toxin to mediate the direct killing of these segregants.

3.4. Example of a Virulence Plasmid from Yersinia pestis

Figure 8 shows the map for virulence plasmid pCD1 from *Y. pestis* (Cornelis et al., 1998). The location of the replication and partition genes are indicated among the large number of genes dedicated to the type III protein secretion system used for virulence of the organism. Very similar plasmids are also found in *Y. enterocolitica* and *Y. pseudotuberculosis* indicating that this organization of genes has been conserved as the plasmid has been transferred to, and/or evolved with, different bacterial hosts.

4. Bacteriophages and Transduction

Bacteriophages (or "phages") are viruses that infect bacterial cells. They consist of a nucleic acid genome (usually DNA, but can also be RNA) that is enclosed within a polymeric protein package called a *capsid* (or "head") (see the phages depicted in Figure 9). Typical phages also possess a multiprotein structure called a *tail* that extends outward from the capsid and serves to interact with the bacterial cell surface during injection of the genome into the host cytosol. Phages bind a host bacterium at the surface via the tail (sometimes with the assistance of tail fibers that extend off the tail), and inject or shoot the genome inside the host where the intracellular part of the phage life cycle commences. As part of this life cycle, the phage DNA may integrate into the host chromosome or remain as an extrachromosomal element. Also, during packaging of the newly replicated phage genomes, the phage may "steal" a large section of the host chromosome and transfer it to a new host bacterium. Thus, bacteriophages are potent machines that can serve to drive the horizontal transfer of genetic material.

4.1. Lytic and Lysogenic Bacteriophages

The life cycle of phages can be divided into two distinct phases or "cycles": the lytic and the lysogenic. In the *lytic cycle*, the phage infects a host bacterium, the phage genes are expressed, new phage genomes are synthesized

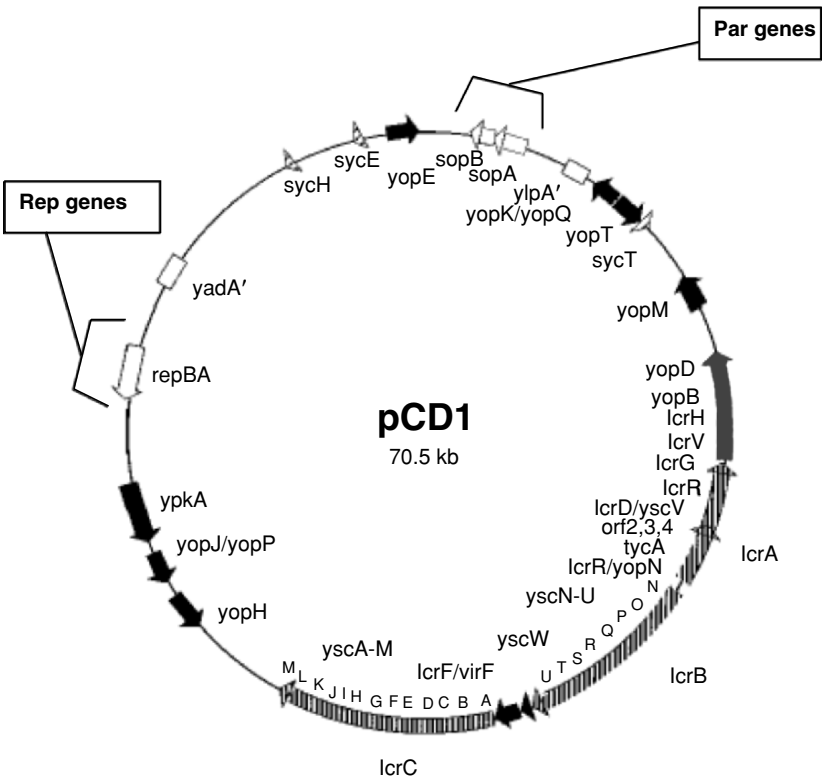

FIGURE 8. Virulence plasmid pCD1 from *Yersinia pestis.* Shown is a map of the virulence plasmid pCD1 from *Yersinia pestis*. The locations of the Rep and Par genes are indicated. The majority of the genes on this plasmid (the *yop*, *ysn*, *syc*, and *lcr* genes) are dedicated to the function of a type III secretion system, which is a major virulence determinant of this bacterium. This genetic structure is conserved on similar plasmids found in *Y. enterocolitica* and *Y. pseudotuberculosis*. (From Cornelis et al, 1998.)

and packaged into newly formed capsids, and the host cell lyses to release progeny phage particles that are able to infect other bacterial cells. This can be thought of as an "infect-replicate-disperse" phase of the phage life cycle. By contrast, the *lysogenic cycle* occurs when the phage genome integrates into the host chromosome, remains there in a "dormant" state (termed a "prophage"), and replicates along with the chromosome. At some point, usually in response to an environmental or cellular signal, the prophage will excise from the host chromosome and initiate a lytic cycle that leads to cell lysis and dispersal of newly formed phage particles. Some bacteriophages are only capable of lytic replication (e.g., the "T-even" phages T2, T4, and T6), while others are able to switch between the lytic and lysogenic cycles (e.g., lambda phage). These two main cycles of phage replication are depicted in Figure 9.

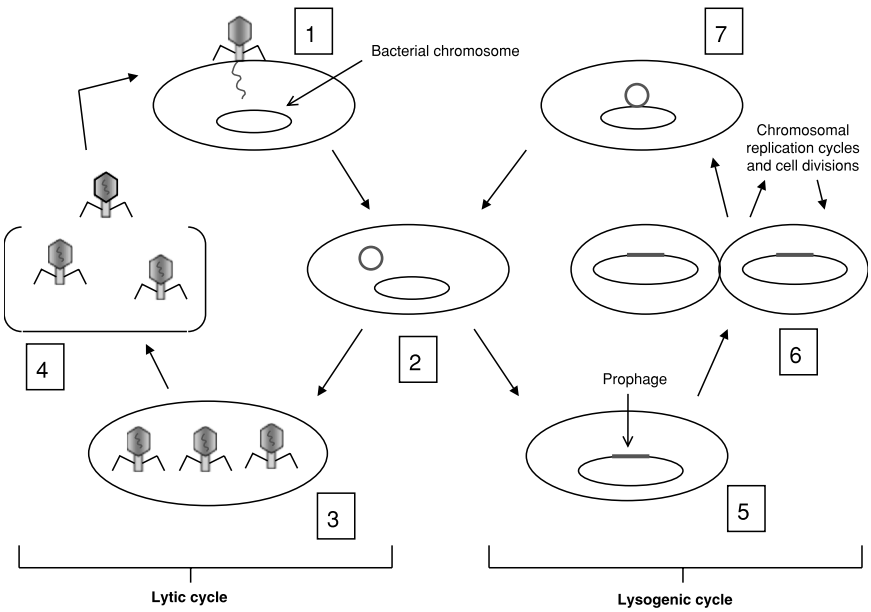

FIGURE 9. The lytic and lysogenic cycles of bacteriophage replication. This diagram shows a single type of phage that can enter either the lytic or lysogenic cycles, but there are phages that are only lytic and do not enter the lysogenic cycle. Also, the phage morphology depicted here shows a capsid, tail, and tail fibers, but other morphologies are common including those with no tail fibers and/or a very short tail. (1) The phage binds to the surface of a host bacterial cell and injects its genome into the cytoplasm. (2) Typically, the phage genome will circularize and the phage enters either the lytic or lysogenic cycle. The expression and stability of the integrase protein at this step is a key determinant of which cycle is entered. If the integrase is expressed and remains stable, this favors the lysogenic pathway. (3) In the lytic cycle, new phage genomes and packaging proteins (i.e. the capsid, tail, and tail fibers) are synthesized and new virions are assembled. (4) Cell lysis occurs and the new phage virions are released. Cell lysis is typically directed by one or more phage-encoded proteins. The newly released virions go on to bind other bacterial cells and initiate more replication cycles. (5) The lysogenic cycle begins with the integration of the phage genome into the host chromosome by the integrase protein (commonly with the assistance of certain host proteins as well). The stably integrated phage genome is termed a "prophage." (6) The prophage is replicated along with the host chromosome. This prophage can remain integrated in the host genome over a very large number of cell generations. (7) At a certain point (usually in response to an environmental or cellular signal), the prophage will excise from the host genome and initiate a lytic cycle.

An important concept to keep in mind when thinking about phages is that upon injection of a DNA phage genome into a host cell, the genome will frequently circularize. This covalently closed circular DNA can then serve as a substrate for a phage-encoded *integrase* protein that catalyzes the insertion of

the phage into the host chromosome (Azaro and Landy, 2002; Van Duyne, 2002). In some cases, the circular phage genome can replicate as a plasmid for several host cell generations before entering the lytic or lysogenic cycle. It is usually the expression and stability of the integrase protein that determines if the phage will enter the lytic or lysogenic cycle. In addition, the DNA site-specificity of the integrase frequently determines where in the host chromosome the circular genome will insert and remain located during the lysogenic cycle (Azaro and Landy, 2002; Van Duyne, 2002).

4.2. Generalized and Specialized Transduction

Transduction is the transfer of bacterial chromosomal DNA between cells via a bacteriophage. There are two main types of transduction: generalized and specialized. In *generalized transduction*, any part of the bacterial chromosome can be packaged and transferred by the phage (Figure 10A). This occurs when pieces of bacterial chromosome are mistakenly packaged into the capsid during phage assembly and are transferred to a new bacterium upon infection by the "chromosomal DNA-carrying" phage particle. The injected chromosomal DNA can be inserted into the new host genome via the endogenous recombination mechanisms. The bacterial host providing the packaged DNA is termed a "donor" and the host which receives that DNA by the phage is termed the "recipient." Usually, the genomes of the donor and recipient in a transduction are highly related. Therefore, the donor chromosomal fragment will integrate at the identical spot in the recipient genome as dictated by the homologous recombination mechanisms. However, any mutation that is present in the donor DNA on that fragment (such as a deletion, point mutation, or transposon insertion) is able to be transferred to the recipient genome.

In *specialized transduction*, only genes that are located near the phage (or "linked") are able to be packaged and transferred (Figure 10B). This happens when a lysogenic prophage excises and, due to a recombination error, carries an adjacent section of the host chromosome with it. The phage genome plus the chromosomal section are packaged in a capsid, transferred to a new host, and both are integrated into the new host chromosome.

The descriptions of generalized and specialized transduction given above show how phages can transfer sections of the host chromosome that have been mistakenly packaged along with the phage genome. It is also important to understand that phages can also transfer genes that have been inserted within the boundaries of their genomes, either by transposons, other phages, or some other kind of recombination mechanism. Several prophages have been identified that contain foreign genes, particularly toxins and virulence factors, which contribute to disease (Table 1) (Novick, 2003). Sometimes, a prophage will become inactivated by mutation over the course evolution and remain inserted at its particular chromosomal location. These prophage "remnants" are becoming increasingly recognized upon sequencing of different bacterial genomes and contribute to the notion that phages play a major role in the horizontal transfer of large blocks of genes (Brussow et al., 2004).

4.3. Regulation of Phage-encoded Toxins by Host-encoded Regulators: Diphtheria Toxin and Cholera Toxin

A striking example of microbial evolution is the regulation of phage-encoded genes by regulator proteins that are encoded outside of the phage genome in the host chromosome. Two classic examples of this phenomenon are illustrated by the regulation of diphtheria toxin encoded by the beta-prophage of

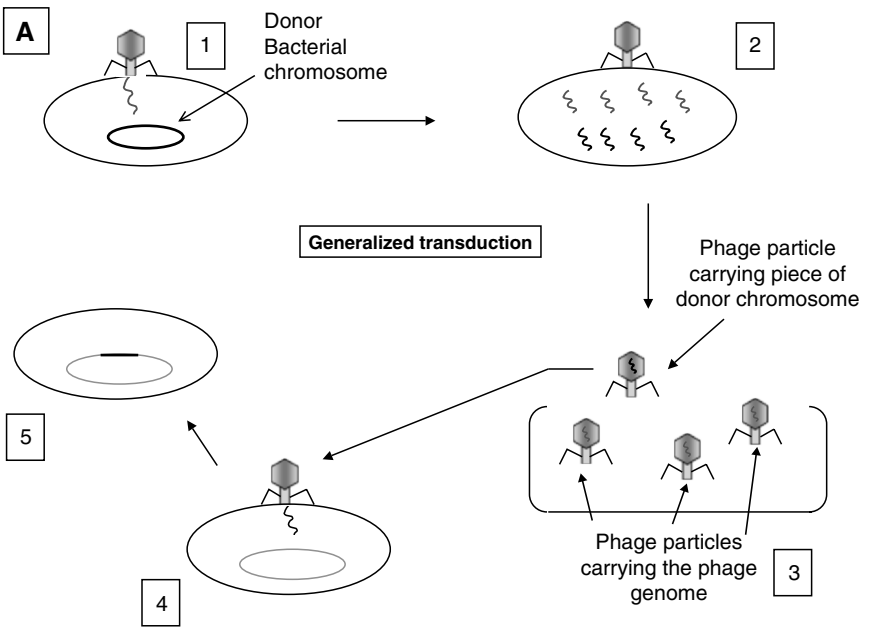

FIGURE 10. Generalized and specialized bacteriophage transduction of bacterial host genes. Panel A: *Generalized transduction*. (1) The phage injects its genome into the host cell, and the lytic cycle of replication is initiated. The infected cells at this point are termed donor cells because genes from their genome will be transferred to a new host. (2) During the lytic phase of certain phages, the host chromosome is fragmented into large pieces prior to cell lysis, and a mixture of newly replicated phage genomes and host chromosomal fragments is produced. (3) The host chromosomal fragments are able to be packaged into phage virions and then transduced to new host cells, termed recipient cells. (4) The phage virion carrying the host chromosomal fragment injects this genomic DNA piece into the new host cell. (5) The injected chromosomal fragment is able to recombine into the recipient host genome by homologous recombination. Usually, the genomes of the donor and recipient in a transduction are highly related. Therefore, the donor chromosomal fragment will integrate at the identical spot in the recipient genome as dictated by the homologous recombination mechanisms. However, any mutation that is present in the donor DNA on that fragment (such as a deletion, point mutation, or transposon insertion) is able to be transfered to the recipient genome. A key feature of generalized transduction is the fact that any donor chromosomal fragment can be packaged by the phage.

(*Continued*)

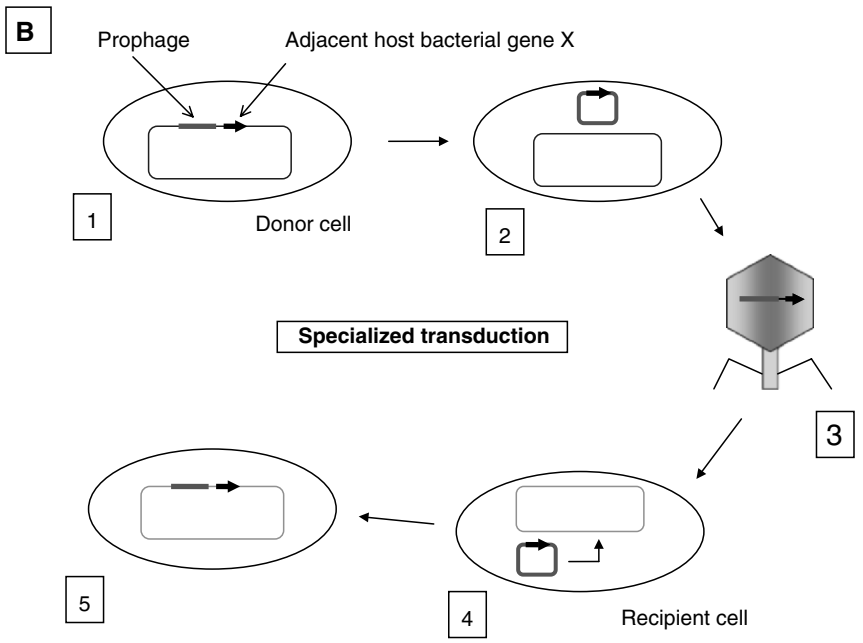

FIGURE 10. (*Cont'd*). Panel B: *Specialized transduction*. (1) In specialized transduction, a host bacterial gene located adjacent to a prophage (termed here "gene X") is transduced to a recipient host. (2) Usually prophage excision from the chromosome is precise and only includes phage DNA with no adjacent host DNA. However, occasional recombination errors occur such that an adjacent host gene(s) is excised with the phage genome. (3) The phage plus host genomic DNA fragment (called a "transducing fragment") is packaged by a phage virion. Typically, excision of the prophage results in a circular DNA molecule, but the phage packages the DNA as a linear molecule. (4) The transducing fragment is transferred to a recipient cell by the phage where it is integrated into the recipient chromosome at its specific integration site. As with generalized transduction, any mutation that is present in the transduced donor gene(s) is able to be transferred to the recipient. (5) The prophage and donor gene *X* are now located in the recipient chromosome.

Corynebacterium diphtheriae and cholera toxin (CT) encoded by the CTX phage of *V. cholerae* (Faruque et al., 1998; Holmes, 2000; Snyder and Champness, 2003). These examples show how genes carried by horizontally transferred elements can evolve to come under the control of host regulatory systems.

The gene encoding diphtheria toxin (termed *tox*) is located on a prophage (beta-prophage), but its expression is regulated by the DtxR protein, which is located elsewhere on the host chromosome (Holmes, 2000; Snyder and Champness, 2003). DtxR is highly related to Fur, the well-characterized iron response regulator in *E. coli*. Not surprisingly, the activity of DtxR is responsive to iron concentrations in a manner similar to Fur. In conditions of high iron concentrations, DtxR binds to ferrous ions (Fe^{2+}) and assumes a

conformation that allows it to bind DNA and act as a repressor of gene transcription. Several genes are repressed by DtxR under these conditions including the *tox* gene on beta-prophage. When iron concentrations drop to low levels (such as in a eukaryotic host environment), DtxR DNA-binding activity drops significantly and repression of the DtxR-regulated genes is lost. These genes, including *tox*, are now able to be expressed under the low-iron conditions where the activity of their products is likely to be advantageous to the bacteria. In this way, the toxin is not expressed outside the host where it is not needed.

Two genes encode the subunits of CT (*ctxA* and *ctxB*), and they are located on the *V. cholerae* CTX prophage. The *ctxAB* genes are regulated by the activity of several proteins (ToxR, ToxS, ToxT, TcpP, and TcpH) that are encoded by genes located elsewhere in the *V. cholerae* genome (Faruque et al., 1998; Snyder and Champness, 2003). The ToxR/ToxS and TcpP/TcpH protein pairs function together to activate the transcription of the gene encoding ToxT as well as other virulence factors. The ToxT protein then goes on to activate the transcription of the *ctxAB* genes. There are DNA-binding sites for ToxR/ToxS upstream of the *ctxAB* genes as well, and the ToxR/ToxS complex also contributes directly to promote *ctxAB* expression. Interestingly, the *toxT*, *tcpP*, and *tcpH* genes are located on another horizontally transferred genetic element, the *V. cholerae* pathogenicity island, termed VPI.

5. Transposons and the Transposition of DNA

Transposons are DNA segments that are able to transpose or "jump" from one genomic location to another. This is accomplished in large part by the activity of a *recombinase* (also called a *transposase*) that catalyzes the insertion of the transposon into a new DNA site. Different transposons may have different insertion site specificities: some insert randomly or near-randomly, while others display insertion site preferences (Craig et al., 2002). Transposable elements are ubiquitous in nature; they are found in cells from all branches of life. In bacterial cells, the transposition of DNA represents a key mechanism by which vertically and horizontally transferred genetic elements can exchange DNA segments. In addition, transposons are frequently used as convenient tools for mutagenesis and have been invaluable in the genetic analysis of many different types of bacterial species (Craig et al., 2002; Hayes, 2003).

Along with the transposase, the activity of a transposon depends on *inverted repeat* sequences that are located on the outside ends of the DNA element. These inverted repeats mark the boundaries of the transposon and are recognized by the transposase during the recombination event that allows transposon movement. Another characteristic of transposons is the presence of *direct repeats* on either end of the transposon, right next to the inverted repeats. The direct repeats represent the site of transposon insertion (or

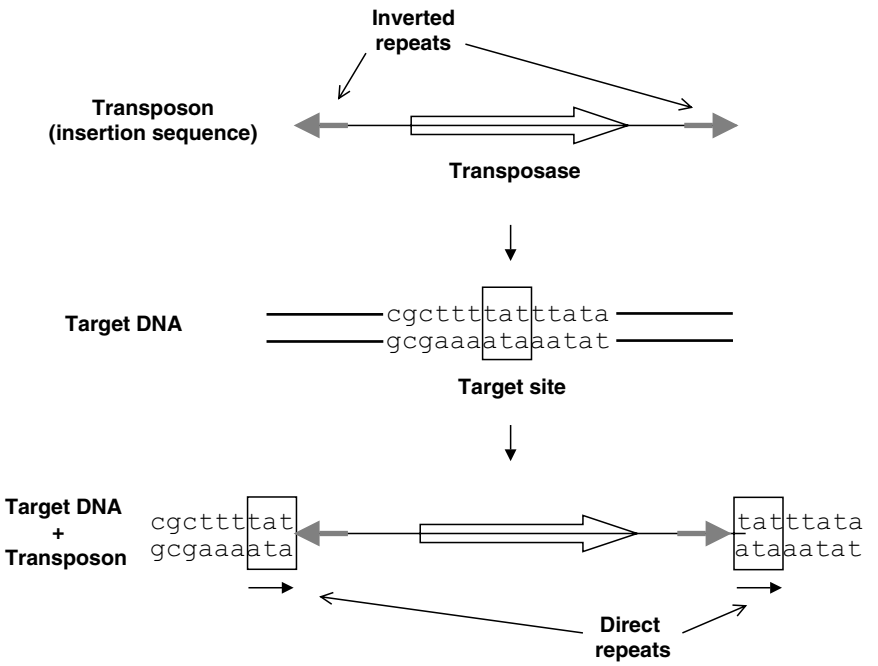

FIGURE 11. Structural features of a transposon. An insertion sequence is depicted as an example of a transposon. Note the inverted repeats formed by each end (that mark the boundary of the transposon) and the internal transposase gene. The inverted repeats vary in size between different transposons but are typically in the range of 15–50 bp. The target DNA contains the target site into which the transposon will insert. This site is duplicated as a result of the transposition reaction.

target site); this site gets duplicated during the recombination reaction. These essential features of transposons are depicted in Figure 11.

5.1. Insertion Sequences, Composite Transposons, and Noncomposite Transposons

The simplest type of transposon is called an *insertion sequence* (or IS element). A typical insertion sequence contains inverted repeats at its ends and a transposase enzyme located in between the inverted repeats (see Figure 12). Insertion sequences do not carry resistance genes and were first discovered because their insertion inactivated certain genes. Although many hundreds of different insertions sequences have been discovered, most of these can be placed in roughly 20 distinct families (Mahillon and Chandler, 1998).

A *composite transposon* is formed when two complete insertion sequences flank other genes, most commonly those encoding resistance to antibiotics.

The activity of the two insertion sequences moves the genes located between them so that the entire composite of two IS elements and associated genes transpose together. Certain composite transposons contain IS elements that are in the same orientation while others have them in inverted or opposite orientation. The composite transposons depicted in Figure 12 have inverted IS elements. An example of a composite transposon with IS elements in the same orientation is Tn9 (not shown in the figure).

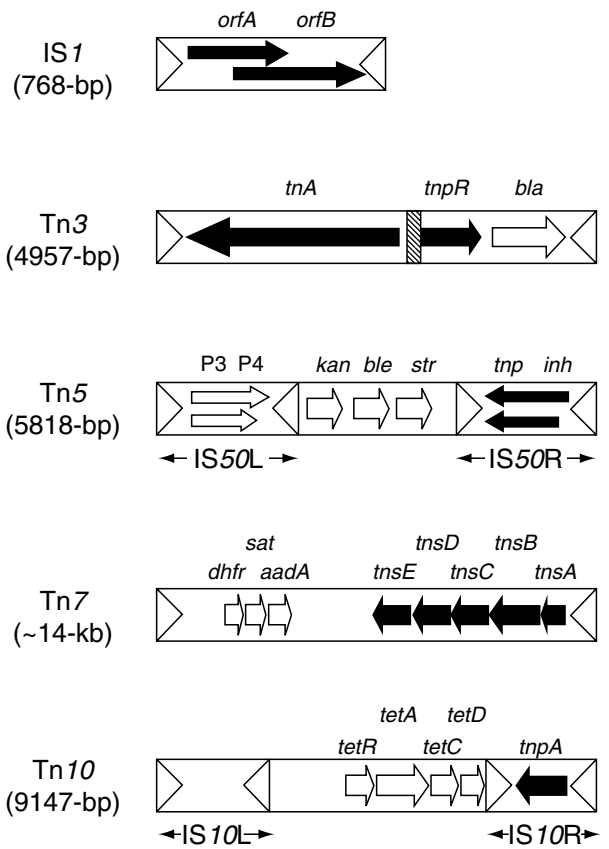

FIGURE 12. Structural organization of selected transposons. Several transposons are depicted as examples of different structural organization of these elements. The inverted repeats are designated as triangles at each end of the transposons, and the genes associated with each element are depicted as arrows. The IS1 element is an insertion sequence and represents the simplest type of transposon. Tn5 and Tn10 represent composite transposons formed by genes that have been "captured" between two insertion sequences. The insertion sequences and associated genes move as one unit during transposition. Tn3 and Tn7 are examples of noncomposite transposons consisting of an insertion sequence that has acquired antibiotic resistance genes between its inverted repeats. (From Hayes, 2003. Reprinted with permission from Annual Reviews, www.annualreviews.org)

Noncomposite transposons can be thought of as a single insertion sequence that contains an antibiotic resistance gene(s) in between its inverted repeats along with the transposase enzymes. Examples of noncomposite transposons are Tn3 and Tn7 as shown in Figure 12.

5.2. Cut-and-paste Versus Replicative Transposition

There are two main mechanisms by which transposons move from one location to another: cut-and-paste and replicative. In *cut-and-paste* transposition, the transposase binds to the ends of the element and brings them together such that the transposon "loops out" of its location. The transposase catalyzes DNA strand cleavage, which releases the transposon as a circular element that is able to integrate into a new DNA site (see Figure 13). A new copy of the transposon is not created during the cut-and-paste mechanism. Examples of cut-and-paste transposons are Tn5, Tn7, and Tn10 (Hayes, 2003; Snyder and Champness, 2003).

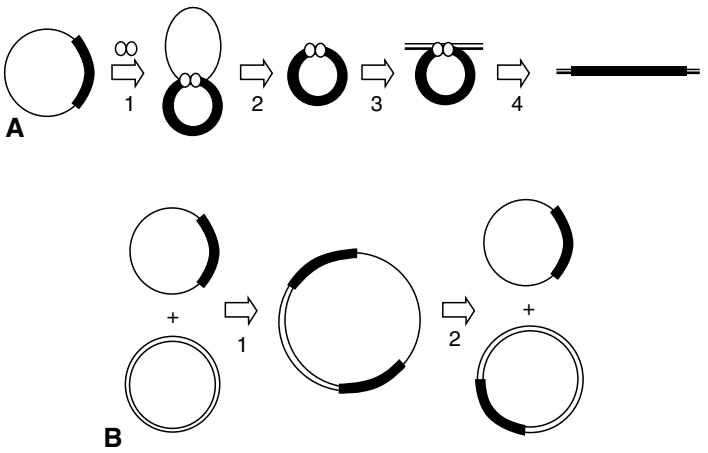

FIGURE 13. Cut-and-paste and replicative transposition. Panel A: *Cut-and-paste transposition*. (1) A transposon residing in a donor DNA molecule is "looped out" by the activity of the transposase enzyme (depicted here as two small circles to represent a dimer). (2) The transposase catalyzes the excision of the transposon from its original location as a circular molecule. (3) The transposase then catalyzes the integration of the transposon into the target DNA molecule. (4) The integrated transposon is depicted. Panel B: *Replicative transposition*. (1) During replicative transposition, a cointegrate is formed between the transposon donor DNA and the target DNA molecules. When the cointegrate is formed, the transposon is duplicated. (2) The activity of a resolvase enzyme catalyzes the resolution of the cointegrate. A copy of the transposon remains in the donor DNA molecule and a new copy now resides in the target DNA. (From Hayes, 2003. Reprinted with permission from Annual Reviews, www.annualreviews.org)

Replicative transposition involves duplication of the transposon and formation of a cointegrate between the donor and target DNA molecules (Figure 13). The resolution of the cointegrate is catalyzed by the activity of a transposon-encoded enzyme called a *resolvase*. This resolution leaves a copy of the transposon in the donor molecule and a new copy in the target molecule. The resolvase is distinct from the transposase; the transposase catalyzes the initial strand cleavage and joining reactions between the donor and target molecules to form the cointegrate, while the resolvase serves to resolve the cointegrate into two separate molecules again. Examples of replicative transposons are Tn3 and the phage Mu transposon (Hayes, 2003; Snyder and Champness, 2003).

6. The Modular Structure of Mobile Genetic Elements

From the descriptions of the major DNA vehicles above, it is clear that their biology involves a number of different functions such as replication, conjugation, phage particle formation, integration, and excision. A useful way to think about these functions is to view them as genetic modules that are combined in different ways to form a given mobile DNA element (Burrus and Waldor, 2004; Osborn and Boltner, 2002; Toussaint and Merlin, 2002). For example, a particular bacteriophage may consist of modules for genome replication, integration, and packaging. Likewise, a given plasmid may contain modules for replication, conjugation, and maintenance. When mobile genetic elements are viewed as combinations of specific modules, it is clear how different elements evolve via the "mixing and matching" of modules. We have explained the basic mobile DNA vehicles above: plasmids, bacteriophages, and transposons. However, nature has evolved many variants of mobile DNA elements. The P1 bacteriophage genome is essentially a large, stably maintained plasmid that encodes packaging functions for movement from host to host. The bacteriophage Mu is a randomly inserting transposon that can be packaged for transfer.

In Section 6.1, we will give some examples of different kinds of mobile genetic elements that represent unique combinations of modules and deviate from typical classification schemes. Below is a list of the different genetic modules that are used in various combinations to create different elements (Burrus and Waldor, 2004; Osborn and Boltner, 2002; Toussaint and Merlin, 2002). These modules are based on functions found in the basic genetic vehicles described above and are illustrated in Figure 14.

6.1. Genetic Modules Found in Mobile DNA Elements

(1) *Replication (Rep)* represents a replicon that drives DNA replication.
(2) *Transfer (Tra)* functions are used for Mpf to allow cell contact and DNA pore generation for conjugative transfer.

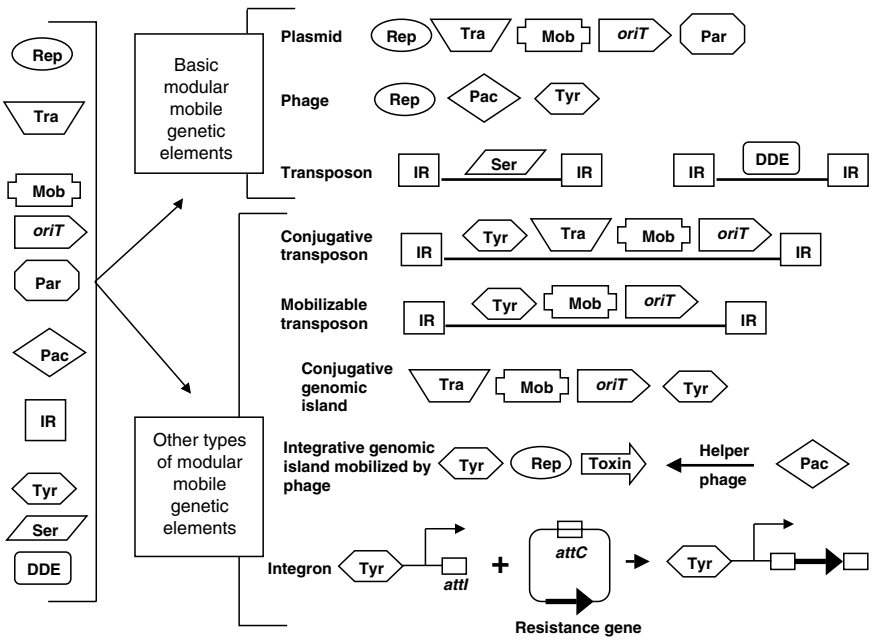

FIGURE 14. Combinations of genetic modules form different mobile DNA elements. This diagram shows the different combinations of genetic modules that make up a variety of mobile DNA elements. In a column on the left side of the diagram, the functions of the different genetic modules are shown: Rep (replication genes), Tra (conjugative transfer genes corresponding to the mating pair formation (Mpf) system), Mob (mobilization genes corresponding to the DNA transfer and replication (Dtr) system that processes the *oriT* for transfer), *oriT* (origin of transfer), Par (genes involved in plasmid maintenance or partition), Pac (genes involved packaging DNA into a phage capsid), IR (inverted DNA repeats), Tyr (tyrosine recombinase typical of bacteriophages and other mobile DNA elements), Ser (serine recombinase found in certain transposons such as Tn3 and gamma-delta), DDE (transposase found in transposons such as Tn7 and Tn10). In different combinations, these genetic modules form the structure of mobile DNA elements that can be divided into two main groups: (1) basic modular mobile genetic elements such as plasmids, bacteriophages, and transposons and (2) other types of modular mobile genetic elements. Details about each mobile element can be found in the text. (Based on concepts presented in Osborn and Boltner, 2002.)

(3) *Mobilization (Mob)* relaxase functions process the *oriT* for conjugative transfer and correspond to the DNA transfer and replication functions (Dtr) indicated above.
(4) *Origin of tranfer (oriT)* is the DNA site on a mobile DNA element recognized by Mob functions for conjugation.
(5) *Maintenance/partition (Par)* functions ensure faithful inheritance and stable maintenance of an autonomously replicating DNA element.

(6) *Packaging (Pac)* genes are necessary for the packaging of mobile DNA into protein capsid particles.
(7) *Inverted repeats (IR)* are DNA sites that are recognized by recombinases for the integration and excision (i.e., transposition) of mobile DNA.
(8) *Recombinases* (*Tyr*, *Ser*, and *DDE*)—*Tyr*: tyrosine recombinase found in several bacteriophages and other mobile DNA elements; *Ser*: serine recombinase (resolvase/invertase) found in certain transposons Tn3 and γδ; *DDE*: typical transposase found in Tn7 and Tn10. Virtually all mobile DNA recombinases can be assigned to one of these families. The reference to tyrosine and serine in the classification of these recombinases refers to conserved amino acids that are involved with the catalytic mechanisms of each enzyme group.

6.2. Other Types of Mobile Genetic Elements that Are Combinations of Modules

These examples of mobile genetic elements consist of different combinations of the modules listed above and in Figure 14. Their discovery and characterization indicate that not all mobile DNA elements fall into standard categories such as plasmid, bacteriophage, and transposon. A common feature of these elements is their ability to integrate into, and excise from, the host bacterial chromosome. In addition, these elements are able to self-transfer or be mobilized from one host to another and then integrate into the genome of the new host. Typically, an extrachromosomal replication phase is not a predominant part of their life cycle; these elements tend to rely on an "excision-transfer-integration" mode of existence.

6.2.1. The ICE Family

A number of these elements have been ascribed different names such as conjugative transposons, conjugative genomic islands, mobilizable transposons, and conjugal, self-transmissible, integrating element (CONSTIN) (Burrus and Waldor, 2004; Osborn and Boltner, 2002; Toussaint and Merlin, 2002). Recently, it was proposed that all such elements fall under the term "ICE," standing for *i*ntegrative and *c*onjugative *e*lement. The term "ICEland" has also been proposed as a fusion between *ICE* and genomic is*land*. We will describe various ICEs below under the descriptive names they have already been ascribed, but it is helpful to think of the different elements as subclasses of ICEs. Hopefully, these examples of mobile DNA elements illustrate the vast number of mechanistic possibilities that exist when genetic modules are put together in different combinations.

6.2.1.1. Conjugative Transposons

Conjugative transposons utilize excision and integration to jump between different genomes (similar to transposons and integrative phages), but with the

added feature that they encode their own conjugation systems that direct their self-transfer from a donor bacterium to a recipient (Churchward, 2002). These elements utilize a circular intermediate that is produced by excision from the host genome and this intermediate serves as a substrate for conjugative transfer. The element recircularizes in the recipient and is then integrated into the new host genome. The integration and excision of conjugative transposons is mediated by tyrosine recombinases that are more similar to those that perform site-specific recombination (similar to phage integration) than those that catalyze movement of typical transposons (like the Ser and DDE recombinases). Conjugative transposons can integrate at different sites in the genome, although these sites have related sequence features that indicate the existence of preferred targets. Two examples of conjugative transposons are Tn916 from *Enterococcus faecalis* and CTnDOT from *Bacteriodes* spp.

Tn916. Tn916 was first identified in the Gram-positive opportunistic pathogen *E. faecalis* and encodes resistance to tetracycline via the *tetM* determinant (Churchward, 2002). Tn916 integration is mediated by a tyrosine recombinase, Int, while excision is mediated by both Int and another recombinase termed Xis; Int is a member of the lambda integrase family, while Xis is a small, basic protein that is very similar to the lambda Xis protein. The target site specificity of Tn916 integration can be considered semi-random since it displays a clear preference for A/T-rich sites with a consensus target of 5′-TT/ATTTT(*N*6)AAAAAA/TA-3′ (where *N* is any nucleotide).

Tn916 encodes its own conjugation system that contains an *oriT* site, which is similar to the nick sites of plasmids F and R6K and functions to transfer a single DNA strand to the recipient. The product of the Tn916 conjugation gene *orf21* is a member of the SpoIIIE/FtsK family of proteins that can function to drive DNA transport between cells. The Tn916 conjugation system displays a broad host range, capable of mediating transfer between a variety of Gram-positive cells and between Gram-positive and Gram-negative cells.

The excision and insertion of Tn916 involves a novel DNA intermediate where 6 bp regions at the ends of the element form a heteroduplex (a double-stranded DNA structure where both strands are nonhomologous) to form a circular molecule. Heteroduplex DNA is also formed upon insertion of Tn916 into its target site. These heteroduplexes are resolved upon replication of the newly inserted Tn916 and its flanking regions. This mechanism explains why the ends of integrated Tn916 molecules are rarely homologous.

CTnDOT. CTnDOT is part of a family of related mobile elements that are widely distributed among natural isolates of different *Bacteroides* spp. and are responsible for mediating rampant spread of antibiotic resistance in these bacteria (Cheng et al., 2001; Whittle et al., 2002). CTnDOT carries the *tetQ* gene, which confers tetracycline resistance. In addition to directing their own self-transfer, the CTnDOT elements also mobilize other transposons termed non-replicating *Bacteroides* units (NBUs), which are discussed below.

Like Tn916, CTnDOT integration utilizes a tyrosine recombinase, Int, that is a member of the lambda integrase family. Excision of CTnDOT requires both Int and a small, topoisomerase-like protein called Exc. Although CTnDOT can integrate at multiple sites, it displays greater site specificity than Tn916, resulting in a range of preferred sites, and is smaller than that of Tn916. The target site consensus for CTnDOT is a 10 bp sequence: 5′-GTTNNTTTGC-3′. A highly homologous sequence is also found at one end of CTnDOT, providing a possible reason why the element is directed to integrate at the consensus target sites.

A remarkable feature of CTnDOT is the fact that tetracycline can induce its excision and transfer several thousand fold. This induction is dependent on a regulatory system encoded by CTnDOT that consists of three genes: *rteA*, *rteB*, and *rteC*. The *rteA* and *rteB* genes are part of an operon containing the *tetQ* resistance gene, and this operon is induced by the presence of tetracycline. The RteA and RteB proteins then mediate the expression of *rteC*, the product of which goes on to regulate the expression of several CTnDOT genes involved in excision and transfer.

6.2.1.2. Mobilizable Transposons

Some excision and insertion-based mobile elements do not encode their own conjugation systems but are able to be conjugatively-mobilized from a donor to a recipient (Churchward, 2002). These elements have been termed "mobilizable transposons". Three major types of these elements have been found, represented by the examples listed below:

Non-replicating Bacteroides Units (NBUs). Along with the CTnDOT conjugative transposons, NBUs are important vehicles of antibiotic resistance transmission among *Bacteriodes* and possibly other Gram-positive species. NBU1 is the best-studied member of this group, though other related NBUs, NBU2 and NBU3, are also found in *Bacteriodes* (Churchward, 2002; Shoemaker et al., 1996). These elements contain an *oriT* site and a nickase enzyme (Mob) that processes the *oriT* for transfer by conjugative functions provided by a coresident conjugative transposon. In addition, DNA vectors that carry the NBU1 mobilization region can be mobilized by the IncP plasmid conjugation system in *E. coli*, indicating its ability to function with different transfer systems. Integration of NBU1 requires IntN1, a tyrosine recombinase belonging to the lambda integrase family, and the target site is specifically located at the 3′ end of a leucine tRNA gene. This target site contains a 14 bp sequence that is also present at one end of NBU1.

Tn4551 from Clostridia *spp.* This element is distinguished from NBUs by the fact that it encodes a serine recombinase, TnpX, belonging to the resolvase family and can integrate at multiple different genomic sites (Lyras and Rood, 2000). It contains an *oriT* and the Mob-encoding gene *tnpZ*, and has been shown to be mobilized by the IncP plasmid RP4. An interesting feature of Tn4551 is that the site used for integration on the transposon is located in the

promoter for the *tnpX* gene encoding the recombinase. When the circular form of Tn4551 is integrated at the target site, the –10 and –35 regions of the *tnpX* promoter become separated from both ends of the integrated, linear transposon. This serves to turn off *tnpX* expression unless it integrates downstream of another properly positioned –35 region sequence or whole promoter. Excision of Tn4551 to its circular form restores the *tnpX* promoter and allows normal TnpX expression.

Tn5398 from Clostridium difficile. This element is unique in that it possesses an *oriT* sequence that allows it to be mobilized by separate conjugation functions, but does not appear to encode its own Mob protein that would nick the *oriT* for transfer (Farrow et al., 2001). It also does not appear to encode a typical recombinase enzyme that would mediate its integration and excision. Analysis of the entire Tn5398 sequence did not reveal any genes with obvious homology to either Mob protein or recombinase genes. It is thought that the coresident conjugative transposon Tn5397 provides these functions for Tn5398 excision, integration, and mobilization.

6.2.1.3. Conjugative Genomic Islands

The conjugative genomic islands contain Tra, Mob, and *oriT* modules that mediate their self-transfer from donor to recipient (Burrus and Waldor, 2004; Osborn and Boltner, 2002; Toussaint and Merlin, 2002). They also typically possess a tyrosine recombinase of the lambda integrase family that mediates integration and excision. However, they are distinguished from conjugative transposons in that they integrate at a specific site in their host genome, commonly at one end of a tRNA or other house-keeping gene, as opposed to multiple target sites. A distinguishing feature of genomic islands is that they are found at their particular integration site in certain strains and absent from this site in other closely related strains (see Chapter 4). The conjugative genomic islands provide an explanation for how at least some of the genomic islands are able to horizontally transfer between host strains.

It is important to realize that conjugative genomic islands and conjugative transposons are very similar mobile elements. The distinction made between these two elements in the literature based on their integration sites helps to categorize different subclasses of ICEs, but it is possible under certain conditions for a conjugative genomic island to integrate at sites other than the common preferential location. Also, a number of conjugative genomic islands were termed "IncJ" plasmids when they were initially discovered, and this was based on certain incompatibility features shared by these common elements (most likely related to common recombinase functions) (Churchward, 2002). However, these elements lack any discernible plasmid replicon, and strains harboring them do not yield a stable, circular extrachromosomal form. Their classification as plasmids is now considered inappropriate and further study has revealed that they are part of the ICE family.

Analysis of different conjugative genomic islands has revealed a remarkable conservation of modular organization and DNA sequence homology between elements isolated from different species. Comparison of the genomes of the elements SXT (*V. cholera*), R391 (*Providencia rettgeri*), R997 (*Proteus mirabilis*), and pMERPH (*Shewenalla putrefaciens*) showed a highly conserved backbone consisting of integration, conjugation, and regulatory functions punctuated by auxiliary sequences that included transposons and antibiotic resistance determinants (Boltner and Osborn, 2004). In addition, all these elements integrate at the same genomic site, the 5′ end of the *prfC* gene encoding peptide release factor 3 (RF3). These observations strongly suggest that these elements, though isolated from different bacterial genera at vastly different geographic locations, have a common ancestral origin.

The SXT element from *V. cholera* is a 100 kb genomic island that contains genes coding for chloramphenicol, sulphamethoxazole, trimethoprim, and streptomycin resistance. SXT and related elements had not been detected in *V. cholera* strains before 1993, but are now present in the vast majority of current clinical *V. cholera* isolates from Asia. Recently, it was demonstrated that the SOS response pathway promotes conjugative transfer of the SXT element in a manner similar to lambda phage induction (Beaber et al., 2004). The "SOS-activated" form of RecA protein facilitates the autocleavage of the SXT SetR repressor protein, which alleviates repression of another pair of SXT genes, *setC* and *setD*. The SetC and SetD proteins then induce the expression of a number of SXT genes required for SXT excision and transfer. Remarkably, one of the agents used to induce the SOS response in these experiments, and thus induce SXT transfer, was the fluoroquinolone antibiotic ciprofloxacin. This indicates that the clinical and agricultural use of SOS-inducing antibiotics may promote the horizontal transfer of antibiotic resistance via the SXT-type elements.

6.2.2. Non-ICE Modular Genetic Elements

As noted previously, the elements described above are termed integrative and conjugative elements or ICEs. Below, we discuss some other examples of modular genetic elements that seem to fall out of the ICE category because conjugation is not an integral part of their description. For clarity, we will put them under the "non-ICE" category.

6.2.2.1. Integrative Genomic Islands Mobilized by Bacteriophages

There are some examples of genomic islands that are mobilized by a helper bacteriophage. These islands are integrated at a specific site in the host genome, but can be excised and transferred from donor to recipient in the presence of a helper transducing phage. These elements are distinguished from pathogenicity genes and islands that have been inserted into the genome of an active bacteriophage and are specifically transduced by this phage as

part of its packaged genome. Instead, the examples discussed here are able to "hitch a ride" in transducing particles formed by unlinked helper phages.

Staphylococcus aureus *Pathogenicity Island 1 (SaPI1).* This element encodes the toxic shock syndrome toxin 1 (TSST-1) and two other newly discovered superantigens termed SEK and SEL. It is part of a large family of similar *Staphylococcal* DNA elements that encode superantigens and are integrated at specific sites in the *S. aureus* chromosome. In the presence of phage 80α, SaPI1 can be excised from its genomic site (assisted by the activity of a phage 80α-encoded excision function) and transduced to recipients at a very high frequency (Lindsay et al., 1998; Novick, 2003). There are two populations of phage particles produced in this event: normal-sized particles containing the 80α genome and smaller particles (about 1/3 the size) containing the SaPI1 island. Upon infection of recipient cells, incoming SaPI1 DNA can be detected in a linear form that immediately replicates, forms circular intermediates, and integrates into the genome at its specific *att* site. Another SaPI, termed SaPI2, is similarly excised and transduced by phage 80α. In the absence of 80α, both these islands are very stable and do not detectably excise and transfer. The SaPI elements represent a remarkable example of how a genomic island can cooperate with a bacteriophage to facilitate its transfer.

Vibrio cholera *VPI.* The toxin responsible for severe diarrhea caused by *V. cholera* infection, cholera toxin (CT), is contained on a filamentous bacteriophage that uses the toxin-coregulated pilus (TCP) as its essential receptor during transduction. The TCP is encoded by the large *Vibrio* pathogenicity island (VPI) that is located on chromosome 1 of *V. cholera*, whose genome consists of two circular chromosomes. It has been proposed that VPI is actually another *V. cholera* filamentous phage and that the TCP serves as the coat for this phage (Karaolis et al., 1999). After this initial proposal, several lines of evidence have cast significant doubt on this hypothesis, and other alternative explanations have been used to explain the nature of VPI (Davis and Waldor, 2003; Faruque et al., 2003). Recently, VPI was shown to be transduced via a helper phage (CP-T1) to four different strains of *V. cholera* and insert into its specific integration site in the recipient chromosome (O'Shea and Boyd, 2002). This result strongly suggests that this may be the preferred mechanism for the horizontal transfer of VPI. It has subsequently been shown that VPI excises from its integration site using two VPI-encoded recombinases, Int and VpiT (Rajanna et al., 2003). This excised form is likely the substrate that is packaged by the helper phage.

6.2.2.2. Integrons

Integrons consist of an integrase gene belonging to the tyrosine recombinase family (IntI), a specific integration site termed *attI*, and a promoter that directs transcription through the *attI* site (Komano, 1999; Recchia and Sherratt, 2002). This basic unit then serves as the platform for integration of

genetic elements called "gene cassettes," which consist of a gene (commonly encoding antibiotic resistance) and an integrase-specific site termed *attC*. Site-specific recombination between the integron and the circular form of gene cassettes occurs at the integron *attI* site and the *attC* site. This leads to the insertion of the gene cassette downstream of the *attI*-associated integron promoter and subsequent expression of the inserted gene. Several gene cassettes are able to be inserted into a single integron. "Super-integrons" have been described that contain a very large number (sometimes hundreds) of gene cassettes, and these elements have been shown to be a source of gene cassettes for smaller integrons. Integrons and super-integrons have been found in a diverse range of Gram-negative hosts and are commonly located within transposons and conjugative plasmids (though super-integrons tend to be more stable components of a host genome and not part of the mobile gene pool). Five major classes of integrons have been defined based on the homology of the integrase genes and *att* sites. The ability of integrons to "capture" antibiotic resistance genes and contribute to their dissemination is thought to have played a major factor in the rapid evolution of multiple resistance observed worldwide.

7. Conclusions—A World of Genetic Modules

If evolution is a race, horizontal gene transfer is one of the strategies that give bacteria a huge advantage in that race. Moreover, bacteria do not rely on one plan for this strategy—many types of different modules contribute to horizontal gene transfer. Genetic modules for DNA replication, conjugation, transduction, integration, and excision have all been combined in different ways to create the many types of genetic elements described in this chapter. Armed with this information, molecular microbiologists now have a deeper appreciation of the genetic flexibility of bacteria and the different possibilities that exist when studying the evolution of different microbes. This becomes especially important as we sequence more and more bacterial genomes and strive to develop new ways to combat harmful bacteria.

Questions to Consider

1. During transposition of a cut-and-paste transposon, replication of the transposon does not occur. How do these types of transposons replicate? Is replication of a transposon (or any type of mobile DNA element) important to its survival?

Cut-and-paste transposons are replicated along with the rest of the genome in which it resides and therefore rely on the replication functions the host organism. Therefore, transposition and replication of cut-and-paste transposons are two separate events. Replicative transposons are replicated during both host chromosomal DNA replication and a transposition event.

Replication of a transposon (or any mobile DNA element) is *the most* important thing to its survival. Therefore, learning about how these different DNA elements propagate themselves is key to understanding their existence.

2. Suppose a new mobile DNA element has been discovered that is able to maintain itself as an extrachromosomal circular molecule, but has the ability to integrate into the host chromosome and be transduced to other hosts. List and describe the genetic modules that are likely contained in this new mobile element.

The genetic modules likely present in this element would be: (1) Rep—for its replication as a plasmid-like element; (2) Par—for its stable maintenance as a plasmid; (3) Tyr recombinase—for chromosomal integration (Tyr because it has phage-like features); (4) Pac—for packaging the element for transduction.

3. Describe the three major components of a conjugation system.

The three components of a conjugation system are: (1) an origin of transfer (*oriT*)—the DNA site on the substrate molecule that is recognized and processed by the relaxase; (2) the DNA transfer and replication proteins (Dtr)—refers to the relaxase enzyme that binds and nicks the *oriT* and the coupling protein that joins the *oriT*/relaxase complex to the conjugal pore formed by the mating pair formation proteins; (3) the mating pair formation proteins (Mpf)—these proteins form the sex pilus and conjugal pore.

4. You have discovered a site between two genes in the fully sequenced *Escherichia coli* genome that serves as the target for integration of different mobile genomic islands. You have recently been given a large number of different, uncharacterized clinical isolates of *E. coli* and wish to determine if islands have integrated into this site in these isolates. What strategies could be used to do this?

Some possible strategies could be: (1) with primers designed to hybridize on either side of the target site, use one of the newly improved strategies for directly sequencing bacterial chromosomal DNA using the DNA from the clinical isolates as a template; (2) with the same target site-flanking primers, perform a PCR using the clinical isolate chromosomal DNA as a template to determine if a high–molecular weight product is formed. This would work best if the inserted islands are less than 15 kb in size and a highly processive PCR amplification enzyme is used; (3) perform a Southern blot with clinical isolate chromosomal DNA that has been digested with restriction enzymes that would give an easily identifiable change in restriction pattern if an island is inserted at the target site.

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Chapter 3

Genomics and the Use of Genomic Tools to Study Pathogenic Bacteria

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Historical Landmarks

1970 Needleman–Wunsch global sequence alignment algorithm is developed (Needleman and Wunsch, 1970).

1977 Maxam–Gilbert and Sanger et al. rapid DNA sequencing methods are developed (Maxam and Gilbert, 1977; Sanger et al., 1977).

1981 Smith–Waterman local sequence alignment algorithm is developed (Smith and Waterman, 1981).

1982 Los Alamos Sequence Library becomes GenBank.

1983 Wilbur and Lipman develop new algorithm for searching large databases (Wilbur and Lipman, 1983).

1988 FASTA search utility is developed (Pearson and Lipman, 1988).

1988 National Center for Biotechnology Information (NCBI) is chartered, as part of the National Institute of Health (NIH), to create and maintain biological databases.

1990 Basic Local Alignment Search Tool (BLAST) is developed (Altschul et al., 1990).

1990 Woese proposes Archaea as third domain of life (Woese et al., 1990).

1992 The Institute for Genomic Research (TIGR) is established.

1992 Wellcome Trust Sanger Institute is established.

1995 First microarray for transcript profiling is described (Schena et al., 1995).

1995 First genome of a free-living organism, *Haemophilus influenzae*, is sequenced (Fleischmann et al., 1995).

1996 Yeast (*Saccharomyces cerevisiae*) genome is completed (Goffeau et al., 1996).

1997 Genomes of two model organisms, *Escherichia coli* and *Bacillus subtilis*, are completed (Blattner et al., 1997; Kunst et al., 1997).

1997 Pfam is developed to analyze protein domains (Sonnhammer et al., 1997).

1998 Microarray is developed to analyze whole genome transcriptional expression in yeast (Eisen et al., 1998).

1998 *Caenorhabditis elegans* genome is completed (Consortium, 1998).

1999 Microarray is used to compare gene content of related Mycobacteria (Behr et al., 1999).

1999 *Drosophila melanogaster* genome is published (Adams et al., 2000).

2000 First plant genome, *Arabidopsis thaliana*, is published (Initiative, 2000).

2000 Draft sequence of the human genome is published (Lander et al., 2001; Venter et al., 2001).

2003 Genomes of more than 120 archaea and eubacteria are published.

1. Introduction

Since the early 1990s, perhaps no scientific field has been more affected by the advancements in technology than biology. As recently as the early 1990s, it took a researcher several weeks to sequence a single open reading frame (ORF) of 1500 bp. In contrast, during the month of October 2000, the Joint Genome Institute (http://www.jgi.doe.gov/JGI_microbial/html/index.html), a division of the US Department of Energy, sequenced the genomes of 15 bacteria.

The technologies that allowed for this advancement were predominantly computer-based. They included automated sequencing machines, fast, inexpensive computers to handle the large amounts of data, and algorithms to rapidly assemble the large data sets into meaningful information. These changes required paradigm shifts in the thinking of biologists and also the import of computer scientists, mathematicians, and physicists into the biological sciences. The new field of bioinformatics developed from these changing needs. Bioinformaticists, scientists with training and experience in biology, computer programming, databases, and how those tools are applied to biological data, have played an important role in translating the information generated by the wealth of sequence information and computer programs for biologists.

These changes in biological research can be best seen by looking at how the first bacterial genome was completed and published. Whole-genome sequencing began with the two workhorses of prokaryotic molecular biology, the enteric bacterium *Escherichia coli* and the soil bacterium *Bacillus subtilis*. Sequencing of these genomes was carried out using the standard technique of the time, *chromosome walking*. In chromosome walking, genomic fragments contiguous to a known chromosomal location are isolated and sequenced; therefore a great deal of cloning and physical mapping must be done before sequencing can begin. Chromosome walking is accurate but slow.

At about this time, Craig Venter and his colleagues at The Institute for Genomic Research (TIGR) were using a fast but controversial technique called *whole-genome shotgun sequencing* to sequence the genome of *Haemophilus influenzae*. In this technique, random clones from *a shotgun library* are sequenced, and the sequences are assembled into contigs (regions of contiguous, overlapping subsequences) using computer analysis. The method is very fast, because little biological input or mapping is needed, but can contain inaccuracies.

It was thought that whole-genome shotgun sequencing would not work for such a large genome because repetitive sequences and the inevitable gaps between contigs would hinder assembly of the complete genome. This proved not to be the case. Sequencing of *E. coli* and *B. subtilis* was initiated in the early 1990s, but the sequences were not completed and published until 1997 (Blattner et al., 1997; Kunst et al., 1997). Using shotgun sequencing, TIGR finished the *H. influenzae* genome in less than 12 months and published it in 1995 (Fleischmann et al., 1995). Whole-genome shotgun sequencing is now the technique most commonly used to sequence a genome. By applying bioinformatic techniques, whole-genome shotgun sequencing has become as accurate as chromosome walking, but is dramatically faster.

Bioinformatics is a new and rapidly changing field, and many of the techniques and analysis methods described today may soon be obsolete. As with any new field, some techniques have become well established while others continue to require improvements. This chapter will discuss those techniques that have become standard and will focus on the overarching theme of what biological information has been uncovered from the analysis of bacterial genomes. This chapter includes a description of how a complete bacterial genome is sequenced, assembled, and finished. Subsequent analysis includes annotating the genome by sequence similarity or using pattern identification with modeling algorithms. We will discuss the pitfalls associated with data generated from computer analyses. This chapter will also present examples of knowledge being gained from the new technologies, such as microarrays and genomic comparisons, that allow researchers to make cross-comparisons within and between genomes. The use of these new technologies in analyzing pathogens will also be presented.

2. Genome Sequencing and Assembly

2.1. Required Resources

Currently, the sequencing and annotation of a bacterial genome requires a large team of scientists and computer programmers and an investment of hundreds of thousands of dollars. In brief, the generation of the complete sequence of a bacterial genome requires: (1) molecular biologists to build the shotgun clone libraries; (2) sequence technicians to prepare the clone DNAs

and load them onto the sequencing machines; (3) programmers and database administrators to implement the software for storing the sequence data; (4) sequence finishers to assemble the contigs from the raw data and resolve inconsistencies in the data; (5) bioinformaticists to identify genes, promoters, and other features of the genome using annotation software; and (6) biologists to edit and correct the raw annotation. Because all of these groups are essential to producing the complete genome sequence, most genome papers have many, many coauthors. Genome sequencing requires a sizable investment in automated sequence machines, robots for colony picking, computers, high-speed networks, and software. Figure 1 outlines the overall process of sequencing, assembling, and annotating a bacterial genome.

2.2. Whole-Genome Shotgun Sequencing

The technique most commonly used for generating genomic sequence data is whole-genome shotgun sequencing, the sequencing of clones of random segments of DNA from the genome. To generate a shotgun library, the entire genome of an organism is isolated, fragmented, and cloned into a vector to generate a pool of plasmids containing random pieces of genomic DNA. Shotgun cloning has been used since the early 1980s for sequencing small DNA molecules such as bacteriophage genomes and plasmids. Since TIGR successfully scaled up the procedure, it is commonly used to sequence entire bacterial and even eukaryotic chromosomes. The drawback of shotgun sequencing is that to obtain complete coverage of the genome, with only a few short gaps, it is necessary to obtain raw sequence data equivalent to about 6–10 times the length of the genome ("6–10× coverage"). This sequencing effort will generate coverage of 95–99% of the genome (Fraser and Fleischmann, 1997; Lander and Waterman, 1988). This will leave only a few short gaps, which can be completed by manual methods (Lander and Waterman, 1988). For example, for a genome of 5 million bp, 10× coverage would require that 50 million bp of genomic DNA be sequenced. If an average read length is 500 bp, 10× coverage is equivalent to 100,000 sequence reads. However, with automated methods, it is cheaper and faster to generate redundant sequence data than to use map-based sequence methods. An example of a single read is shown in Figure 2.

2.3. Creating a Shotgun Library

The first step in sequencing and assembling a microbial genome is to construct plasmid libraries containing genomic DNA. Normally, shotgun libraries are constructed with inserts of two size ranges: one library containing DNA fragments of 1–2 kb and a second library containing fragments that are usually 6–10 kb, although libraries containing inserts of 50 kb are often used.

The DNA for insertion is usually generated by random mechanical shearing, after which the ends of the sheared fragments are repaired and ligated

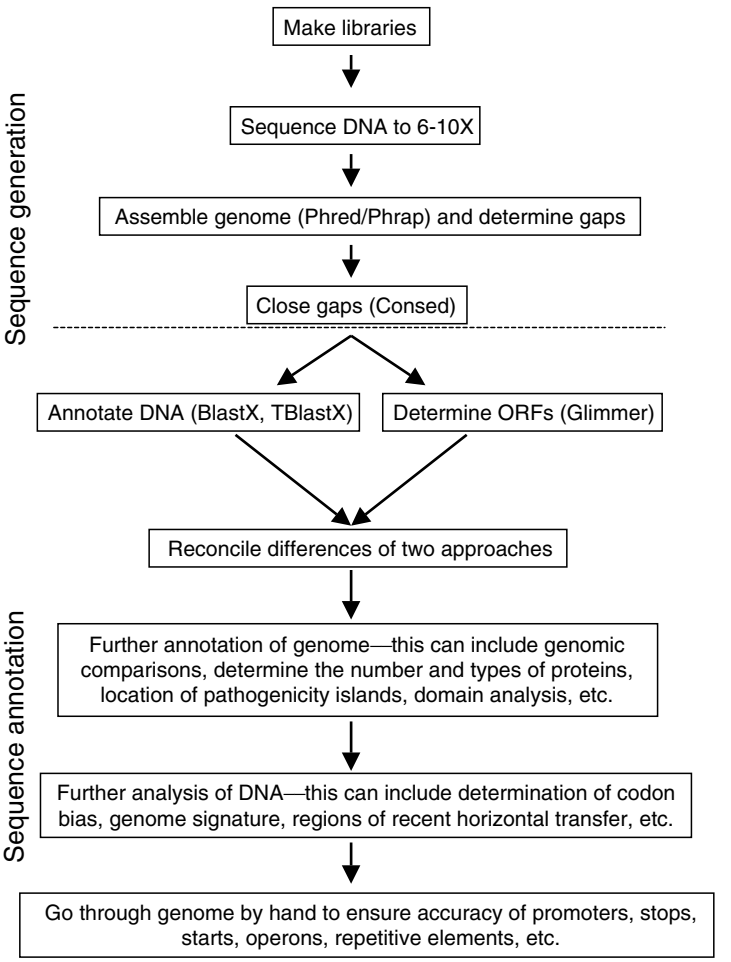

Make libraries
Sequence DNA to 6-10X
Assemble genome (Phred/Phrap) and determine gaps
Close gaps (Consed)
Sequence generation
Annotate DNA (BlastX, TBlastX)
Determine ORFs (Glimmer)
Reconcile differences of two approaches
Further annotation of genome—this can include genomic comparisons, determine the number and types of proteins, location of pathogenicity islands, domain analysis, etc.
Further analysis of DNA—this can include determination of codon bias, genome signature, regions of recent horizontal transfer, etc.
Sequence annotation
Go through genome by hand to ensure accuracy of promoters, stops, starts, operons, repetitive elements, etc.

FIGURE 1. Overview of sequencing and annotating a bacterial genome. DNA fragments from the genome are shotgun-cloned into a vector. The fragments are sequenced, the raw sequences are edited, and poor-quality sequences are removed. To finish, or close, the genome, the edited sequences are assembled into contigs, and gaps between the contigs are closed. The genome is annotated by several high-throughput methods, including using both sequence comparisons and gene prediction algorithms. After annotation the genome must be manually edited and the results reconciled, a process called *curation*.

into a small plasmid vector. The ligation products are used to transform *E. coli*, and the transformed cells are plated under antibiotic selection. Robot colony pickers then identify and pick transformants into 96-well or 384-well plates containing growth medium. The cells are allowed to grow, a cryoprotectant such as glycerol is added, and the cells are frozen for long-term

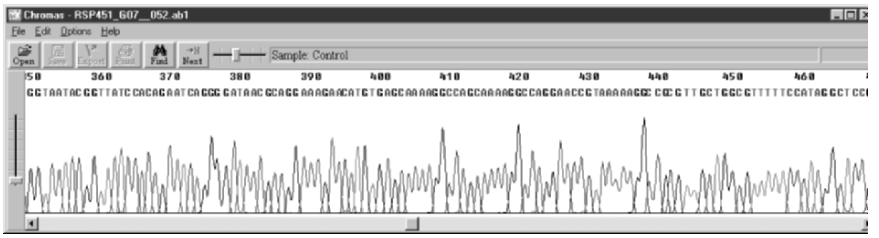


FIGURE 2. A DNA sequence "Trace" file. Example of the output obtained from an ABI-Prism 3700 automated DNA sequencer. (See color insert.)

storage. Each library must be checked to ensure that the average fragment length is as desired and that most of the clones contain inserts.

2.4. Generating Sequence Reads

Once a suitable library has been generated, the cloned DNA is sequenced from both ends using standard primers located in the flanking vector sequence. The bulk of the sequence data is obtained from the library containing the 1–2 kb genome fragments. A smaller amount of sequence data is obtained from the library of larger fragments, and these data are used during the assembly process as a scaffold for the smaller sequences. Sequencing continues until 6–10× coverage is obtained.

The data can be stored in a number of formats, including raw data files, termed "*flat files*." However, these data are most efficiently accessed only in a relational database management system (RDBMS). Computer programmers, database administrators, and biologists must collaborate to develop and implement the sequence database.

2.5. Assembling the Sequence of the Genome

Next, the shotgun sequences are assembled into contiguous regions (termed "*contigs*").The individual sequence reads are collected in a computer, where they are all aligned with one another to assemble contigs. Phil Green and his colleagues at the University of Washington have developed several computer algorithms and tools for assembling genomes, including *Phred*, *Phrap*, and *Consed* (Ewing and Green, 1998; Ewing et al., 1998; Gordon et al., 1998). Many other assembly tools are also available and in use.

Before an individual sequence read can be incorporated into the assembly, the read must be edited. The Phred program calculates an error probability for each base in the read, and the ends of the read are trimmed to remove regions containing bases with high error probabilities. The resultant sequence is then trimmed to remove any vector sequence.

At this point, the sequence read is added to the growing assembly by alignment. As sequences are aligned and the assembly grows, the error probability

at each position is recalculated. The standard for complete genomes is an error rate of less than one base error in 10,000 bases. Phrap also incorporates information about the physical structure of each read into the assembly and uses this information to detect and correct inconsistent assembly; this procedure is termed *scaffolding*. Because long repeated sections of DNA, such as rRNA operons or transposons, can confuse the assembly reads from the shorter 1–2 kb clones, the reads from the longer 10 kb clones provide a scaffold that should span such repeated regions.

Even with 10× coverage and scaffolding, many gaps and inconsistencies remain in the assembly. Closing of gaps and reconciliation of the inconsistencies, termed *finishing*, is a tedious and labor-intensive process. Gaps are eliminated by a variety of techniques, including directed sequencing by chromosome walking. Inconsistencies are investigated and resolved by experienced and highly skilled researchers called sequencing finishers. The finishing process is simplified using an interactive finishing program such as Consed. Consed was developed as part of the assembly process rather than as a standalone editor. As a consequence, it is not only a visual alignment tool but it also incorporates the error probabilities into those alignments. These probability scores are particularly important for the final assembly of the genome. Most assembly programs are particularly sensitive to a large number of repeated sequences. Because many organisms, particularly enteric pathogens, have many repeated regions due to insertion sequences, final assembly can be confounded. Alignment editors such as Consed, which use the probability scores, allow the differentiation of these repeated regions and are critical for finishing the genome.

3. Genome Annotation

"The value of the genome is only as good as its annotation. It is the annotation that bridges the gap from the sequence to the biology of the organism."

Lincoln Stein (2001)

3.1. Identification of Open Reading Frames

The first step in annotating a newly sequenced genome is to use automated software to identify the ORFs. Annotation programs use two broad techniques: similarity-based identification and pattern-based identification.

The similarity-based process identifies genes by performing similarity searches against established sequence databases; these searches use high-speed tools such as Basic Local Alignment Search Tool (BLAST) (Altschul et al., 1990). The BLASTX algorithm translates the DNA into six protein sequences, one for each of the six reading frames (three forward frames and three reverse frames), and compares the sequences to a set of protein sequences from other organisms. Proteins are used as the database standard

for two reasons: there is more information content in an amino acid sequence than in a DNA sequence (because there are 20 amino acids but only 4 nucleotides), and protein sequences are more highly conserved than their underlying DNA sequences.

The pattern-based process determines gene locations using statistically defined patterns typically found in gene boundaries to identify the ORF. The Glimmer (Salzberg et al., 1998) and GeneMark (Borodovsky and Peresetsky, 1994) algorithms use sophisticated pattern models to identify putative ORFs.

After both analyses have been done, their results must be reconciled. The homology- and pattern-based algorithms will find most of the same reading frames. However, the BLAST algorithm has difficulty finding the beginnings and ends of genes where amino acid similarity is not as highly conserved. On the other hand, programs like Glimmer and GeneMark have difficulty defining small genes (<100 amino acids) as well as in identifying genes that have a codon usage and third codon bias that is different from the rest of the genome. As a consequence, genes are often overpredicted by pattern-based methods, and reconciliation of the results is best performed by a biologist, although computer programs are currently being developed to aid this function.

3.2. Identification of Other Elements

After the putative coding regions have been identified, other genetic elements encoded in the DNA sequence must be obtained. These elements include regulatory sequences such as promoters, transcription factor binding sites, and transcription terminators. They also include repetitive elements; insertion sequences and transposons; the sites for the origination and termination of DNA replication; and functional RNAs such as tRNAs, rRNAs, snoRNAs (small nucleolar RNAs), and other small regulatory RNAs. These methods use both similarity- and pattern-based algorithms, and much work has gone into identifying promoters and transcription factor binding sites.

3.3. Protein Domain-based Annotation

A recent technique for determining the function of new genes relies on identifying similarity to well-characterized protein domains, which may consist of structural or functional domains. The domains are specific regions of a protein, are often associated with a biochemical function, and have been conserved in many other proteins. The Pfam (http://pfam.wustl.edu) website provides families of related sequences. Other sites also have protein families. In Pfam, a model or profile of each protein family is generated by aligning the sequences and assigning a statistical weight to each position of the profile (Hidden Markov Model or HMM) (Sonnhammer et al., 1997). These models are then used to identify other members of the family. The putative proteins from the genome are then compared to these databases by using algorithms that incorporate the alignment model; the comparison shows

which domains these proteins contain. Often proteins with low sequence similarity to any protein of known function can be classified this way. In fact, whole groups of proteins have been classified as containing domains of unknown function (DUFs). Other databases that use domain analysis include Prints (http://www.bioinf.man.ac.uk/dbbrowser/PRINTS), ProDom (http://prodes.toulouse.inra.fr/prodom/doc/prodom.html), Prosite (http://www.expasy.ch/prosite), and Simple Modular Architectural Research Tool (SMART) (http://smart.ox.ac.uk/). By combining many approaches it is possible to improve the overall annotation of a genome.

3.4. Metabolic Pathway-based Annotation

Another method of annotation utilizes some of the codified pathway databases that are now available. Many biochemical pathways are conserved between different organisms. By mapping the genes and their putative ORF annotation back to a known pathway database (Dayhoff et al., 1983) such as KEGG (http://www.genome.ad.jp/kegg) or Ecocyc (http://biocyc.org/ecocyc/), one can improve overall annotation. If it is known that an organism synthesizes tryptophan, for example, then all of the genes required for tryptophan biosynthesis must be present. If one of the genes is not present, the annotator looks for another gene that should perform this function.

The entire protein contents of a genome can be used to make a partial metabolic reconstruction of the organism. Figure 3 shows a metabolic reconstruction of the causative agent of syphilis, *Treponema pallidum* (Fraser et al., 1998). In this intuitive display, many pathways such as transport systems, energy production, and murein biosynthesis pathways give a general overview of some of the biochemical pathways in a genome.

3.5. Annotation Error

As of October 2003, 122 bacterial genomes had been completed and added to the public DNA sequence databases, and over 225,000 ORFs have been identified. An additional 200 genomes will be completed in the next few years. The predicted proteins of many genomes have been determined by high-throughput annotation. It must be remembered, however, that the functions of only a fraction of these proteins have been experimentally determined. As a consequence, there are many errors in the annotations of these proteins. The great lament of many researchers is that the annotations associated with genomic sequences often contain inaccuracies. The sources of these inaccuracies fall into two broad categories: problems in annotation methodology and problems in database dependency. Incorrect annotation of proteins based on methodologies falls into three categories: improper gene models, improper algorithm implementation, and human or computer errors. Errors of database dependencies refer to annotation errors that are propagated from one database to the next.

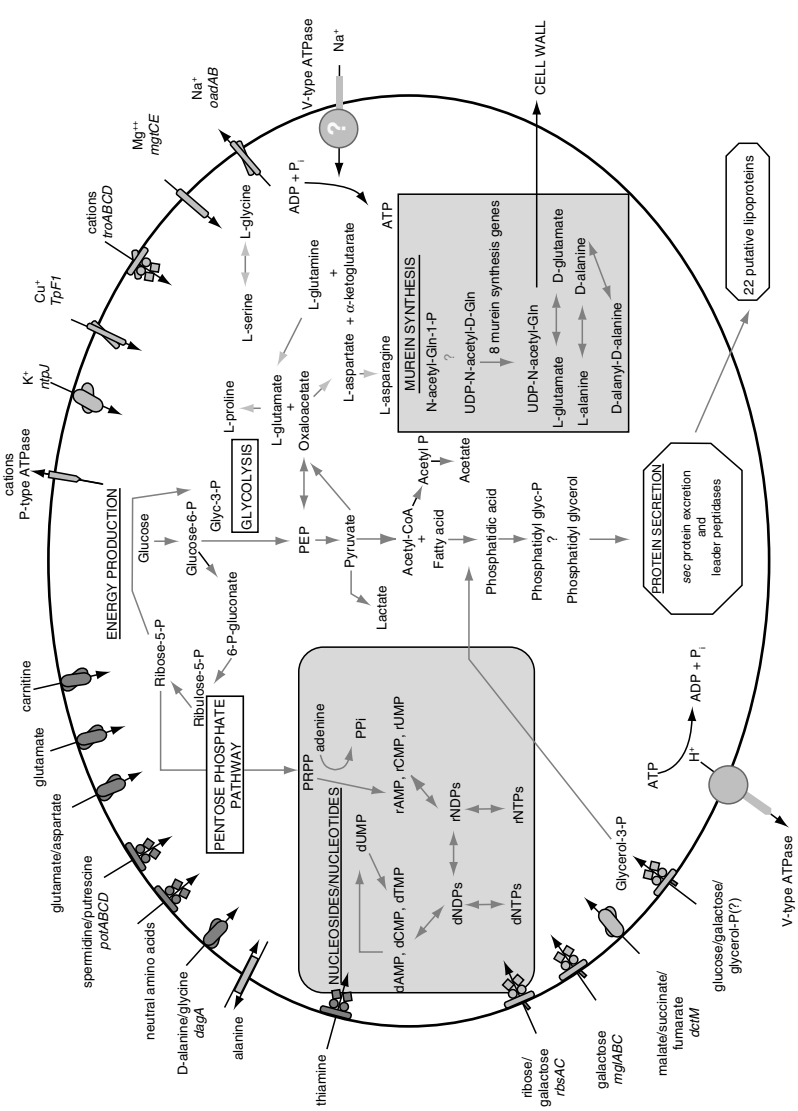


FIGURE 3. Solute transport and metabolic pathways in *Treponema pallidum*. A schematic diagram of a *T. pallidum* cell providing an integrated view of the transporters and the main components of the metabolism of this organism, as deduced from the genes identified in the genome. Presumed transporter specificity is indicated. Question marks indicate where particular uncertainties exist or expected activities were not found. Abbreviations: r, ribo; d, deoxy; AMP, adenosine monophosphate; CMP, cytosine monophosphate; NDP, nucleotide diphosphate; NTP, nucleotide triphosphate; TMP, thymidine monophosphate; UMP, uridine monophosphate; ADP, adenosine diphosphate; CoA, coenzyme A; UDP, uridine diphosphate; PRPP, phosphoribosyl-pyrophosphate.

3.6. Problems in Annotation Methodology

Improper gene models can occur for a number of reasons. The most common mistake is if the protein function of a particular gene product was incorrectly annotated by similarity to a homolog of known function. It is generally assumed that the homologs that are most similar are functional homologs. While this is usually correct, it should never be assumed. In other cases, an incorrect gene model was generated by sequence errors or by the improper determination of the start or stop codons. Sequence contamination also plays a role in improper gene models, because sequences from organisms other than the organism of interest can enter the database. These sequences have no labels to distinguish their origin, so such sequences can be difficult to identify. Often these sequences come from the vector or the clone host, *E. coli*. Improper algorithm implementation can also generate annotation errors. Since most annotation is obtained via high-throughput analysis, the goal is to maximize the annotation of the predicted protein sequences without sacrificing quality. This is done by developing and using computer algorithms such as BLAST and GeneMark. If the computer output has not been checked carefully, errors can be generated unknowingly. Other problems include incorrect annotation based on incorrect setting of the algorithm (e.g., annotation based on homology of only one domain of a multidomain protein). Finally, since there are only about 1,000 protein families, annotation by similarity to members of a family can lead to a correct family designation but incorrect substrate specificity.

3.7. Removing Annotation Errors

The Swissprot database (http://www.ebi.ac.uk/swissprot/index.html) contains well-curated protein sequences. One of the goals of Swissprot is to curate new and existing protein sequence data and clean up misannotated ORFs. DNA sequence data that are generated by public sequencing projects enter GenBank and EMBL, GenBank's European equivalent. The coding sequences are translated and the protein sequences added to the Translated EMBL (Trembl) database. The Swissprot team curates the sequences of the Trembl database using both literature and computer analysis to improve or correct protein and ORF annotation. Once a set of proteins has been curated, the annotation associated with the sequences is added to the Swissprot database. After Swissprot has been updated, the EMBL and Genbank databases are updated with this information. Thus, Swissprot contains a set of curated proteins likely to be correctly annotated, the Trembl database contains uncurated proteins, and Genbank and EMBL contain both data sets. Because sequence data are generated much faster than data can be analyzed and annotated, error-prone high-throughput annotation is the predominant form in most databases.

4. Microarray Technologies

Identifying all the genes of a bacterium through genomic sequencing and analysis is only the first step to understanding their functions. In fact, the genes that are unique to a species or that confer on that species a phenotype of interest are often those that are initially annotated only as "hypothetical protein." Experiments that allow scientists to understand the function of such genes can be difficult to implement for all the genes in an organism.

Many bacterial genes are coregulated in response to an external stimulus, and one step toward understanding gene function is to determine how such genes are regulated by that stimulus. Historically, the transcriptional regulation of individual genes was monitored using fusions to reporter genes or by directly assaying the level of transcription from a specific promoter. However, once an entire bacterial genome has been sequenced, it is also possible to profile the level of transcription of all the genes of the genome using microarray technology (Cummings and Relman, 2000; Lockhart and Winzeler, 2000; Rappuoli, 2000). Transcriptional profiling using microarrays allows the entire gene complement of an organism to be tested for transcriptional expression patterns.

4.1. Transcriptional Profiling Methodologies

The rationale of most transcriptional profiling experiments is to measure the level of transcriptional activation (or repression) for a given set of genes under different, well-defined environmental conditions. Although the specific criteria vary for each experiment, the standard transcriptional profiling experimental protocol is as follows (Figure 4). Total RNA is isolated from bacteria grown under the test (experimental) condition and from bacteria grown under the control condition. The RNA from each bacterial culture is used as a template for the synthesis of a population of labeled cDNAs. The labeled cDNA population is purified and hybridized to an array of spotted DNA molecules, where each spot contains DNA specific to an individual gene of the array, and the intensity of the label is determined for each spot. For each spot, the intensity of the label of the cDNA from the experimental bacteria is compared to the intensity of the label of the cDNA from the control bacteria to determine the relative abundance of mRNA for the gene represented in the spot for each gene. Thus, transcriptional profiling allows the quantitation of gene transcript accumulation of potentially every gene of a genome.

The arrays are created by spotting DNA onto a solid support (e.g., glass slides or nylon membranes) (Figure 4). The DNA spotted on the array may range in length from gene-specific oligonucleotides of as short as 25 bases to full gene sequences. For example, Richmond et al. (1999) created *E. coli* microarrays by spotting PCR-amplified DNAs representing the full coding sequences of 97% of the 4,290 different annotated open reading frames.

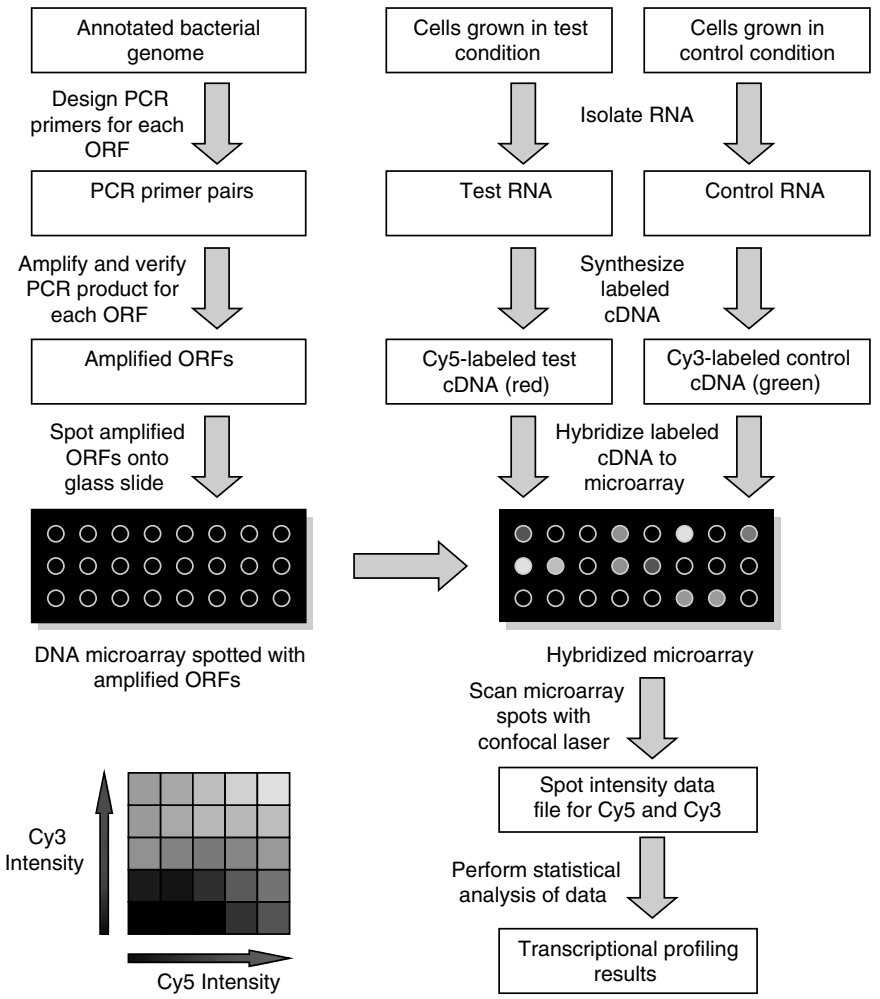

Annotated bacterial genome
Design PCR primers for each ORF
PCR primer pairs
Amplify and verify PCR product for each ORF
Amplified ORFs
Spot amplified ORFs onto glass slide
DNA microarray spotted with amplified ORFs
Cells grown in test condition
Cells grown in control condition
Isolate RNA
Test RNA
Control RNA
Synthesize labeled cDNA
Cy5-labeled test cDNA (red)
Cy3-labeled control cDNA (green)
Hybridize labeled cDNA to microarray
Hybridized microarray
Scan microarray spots with confocal laser
Spot intensity data file for Cy5 and Cy3
Perform statistical analysis of data
Transcriptional profiling results
Cy3 Intensity
Cy5 Intensity

FIGURE 4. Schematic diagram of a transcriptional profiling experiment using a DNA microarray. The diagram follows the experimental design of Richmond et al. (1999) from their investigation of gene expression in *Escherichia coli* K-12. On the left side, the DNA microarray is created by spotting PCR-amplified open reading frames (ORFs) from the annotated genome onto a glass slide. The clear glass slide is shown in black to provide contrast with the DNA spots. On the right side, total RNA is isolated from test and control cultures. cDNA containing Cy5- or Cy3-labeled nucleotides is synthesized and hybridized to the microarray. The intensities of the Cy5 and Cy3 labels are measured using a confocal laser and the data stored in a computer file. The lower left of the figure shows the grid of colors produced by the combinations of Cy5 (red) and Cy3 (green) intensities. Finally, a statistician analyzes the data to produce the experimental results. (See color insert.)

Production line methods and robotics have simplified the arraying procedure so that hundreds of identical copies of the arrays can be created. Since the solid support is small and the DNA spots are small and close together, these arrays are called microarrays.

After the isolation of total purified RNA from bacterial cells, reverse transcriptase—an enzyme that can synthesize DNA from an RNA template—is used to create labeled cDNA. Synthesis of cDNA from bacterial RNA is primed using random hexamer oligonucleotides. The cDNA molecules are usually labeled by the incorporation of labeled nucleotides. Nucleotides containing fluorescent labels (e.g., Cy5 or Cy3) (Gill et al., 2002; Guckenberger et al., 2002; Richmond et al., 1999) or nucleotides containing a radioisotope (e.g., [α-^{33}P]dCTP) (Richmond et al., 1999; Tao et al., 1999) can be used for labeling. Alternatively, cDNA molecules can be labeled by the addition of biotin-N6-ddATP to the 3′ end using terminal transferase (Rosenow et al., 2001).

After cDNA synthesis, the RNA is degraded using NaOH at 65°C (Gill et al., 2002) or using DNase-free RNase (Tjaden et al., 2002), and the labeled cDNA is recovered by precipitation or through the use of a PCR purification kit. Because random primers are used, the length of the cDNA molecules is on the order of 100 bases.

The labeled cDNA is then hybridized to the spots on the microarray, after which the microarray is washed to remove unbound labeled cDNA. The level of hybridization at each spot is determined by measuring the amount of label that is bound to each spot on the array. Radiolabeled arrays are exposed to a phosphor screen, which is scanned by a phosphoimager to determine spot intensities (Richmond et al., 1999). Fluorescently labeled arrays are scanned with a confocal laser scanner (Richmond et al., 1999). Biotin-labeled arrays are incubated sequentially with streptavidin and then antistreptavidin antibody, followed by incubation with streptavidin-phycoerythrin; the fluorescence of phycoerythrin is measured using a confocal laser scanner (Rosenow et al., 2001).

4.2. Sources of Variability

There are two major sources of variability in transcriptional profiling: biological and process. Biological variability is simply culture-to-culture variability, which can have an environmental or biological source. Sources of process variability include the manufacturing of the array; the isolation and purification of total RNA; the labeling of the cDNA; the hybridization of the cDNA to the microarray; and the detection and quantitation of the signal from each spot. Microarray experiments must be carefully designed and executed to minimize these sources of variability. The best experiments contain several biological replicates as well as several samples of the same biological material (process replicates), so that an analysis of variance can be carried out. Because microarray analysis is expensive and the biological materials difficult to obtain, the desired number of replicates is rarely achieved.

4.3. Statistical Analysis of Microarray Data

Statistical methods are applied to microarray data to determine what changes in transcription are significant and which genes are coregulated under a given experimental condition. The analysis of microarray data is best performed by a statistician experienced in *multivariate* statistical methods and using sophisticated statistical software. Fortunately, the abundance of microarray data and the difficulty of interpretation of the results have attracted a number of statisticians to biology. The number of papers describing statistical approaches for analyzing microarray data is growing rapidly. A flavor of the statistical methods required for the analysis of transcriptional profiling data can be obtained by reading the analysis of the light-to-dark transition in *Synechocystis* sp. strain PCC 6803 by Gill et al. (2002).

4.4. Microarray-based Findings

DNA microarrays are becoming a popular and powerful tool for the study of bacterial pathogenesis. Applications of DNA microarrays to study this process include monitoring global changes in bacterial gene expression profiles during (1) the host–pathogen interaction, (2) in vitro growth conditions relevant to those encountered during infection; and (3) treatment with antimicrobial drugs. DNA microarrays are also being used in genomic comparisons of bacterial pathogens to measure differences in DNA content between strains as an indicator of evolutionary relatedness. Because different strains of bacterial pathogens often vary in their ability to cause disease, researchers are using microarrays in comparative genomics studies to uncover the strain-specific basis for diversity and severity of disease. Thus, the use of DNA microarrays to study bacterial pathogenesis is uncovering candidate virulence factors, novel aspects of pathogenesis, and new targets for vaccine design. Microarray technology provides a powerful tool for the diagnosis, prognosis, and clinical management against infectious disease.

Transcriptional profiling experiments have now been carried out in a wide number of bacterial species, and we briefly describe here a few examples. Basic transcriptional profiling experiments in *E. coli* have explored changes in gene expression in cells exposed to heat shock (Richmond et al., 1999), cells grown in the presence or absence of IPTG (Richmond et al., 1999), cells grown in minimal or rich media (Tao et al., 1999), and cells grown in the presence or absence of tryptophan (Khodursky et al., 2000). Tjaden et al. (2002) used a whole genome array to search for transcripts in intergenic regions and found 317 novel transcripts in *E. coli*.

Transcriptional profiling has been used to identify novel genes of *Staphylococcus aureus* that are regulated by the *agr* and *sarA* loci, which are known regulators of the virulence response in that organism (Dunman et al., 2001). Microarray analysis of transcription during the heat shock response in *Neisseria meningitidis* identified 34 induced and 21 repressed heat

shock–regulated genes (Guckenberger et al., 2002). Staudinger et al. (2002) studied the transcriptional response of *E. coli* cells that had been ingested by normal and phagocyte oxidase–deficient human neutrophils and found that genes regulated by *oxyR*, which encodes an oxidant-sensing transcription factor, were induced after ingestion by normal neutrophils but not by the oxidase-deficient neutrophils.

Space considerations allow us to describe in detail a single report of transcriptional profiling in pathogenic bacteria. Dietrich et al. (2003) studied transcriptional changes in *N. meningitidis* during infection of human epithelial cells and human endothelial cells. This experiment used oligonucleotide DNA microarrays carrying the 2,158 annotated genes of *N. meningitidis* serogroup B strain MC58 (Tettelin et al., 2000). In the infection experiment, epithelial cells (HeLa cells) and endothelial cells (human brain microvascular endothelial cells) were infected with *N. meningitidis* at a multiplicity of infection of 10 and incubated for 6 h, after which cell-associated bacteria were isolated and total RNA extracted. Control RNA was isolated from bacteria incubated for 6 h in cell culture medium in the absence of human cells. When *N. meningitidis* infected epithelial cells, significant changes in the level of transcription of 72 genes were found. These genes included membrane proteins and transporters, regulators of gene expression, and enzymes; 15 of these genes were previously identified virulence genes, whereas 20 genes were at the time annotated as hypothetical open reading frames.

Transcription of 48 genes changed when *N. meningitidis* infected endothelial cells (Dietrich et al., 2003). Most of these genes encode virulence factors, transport proteins, or enzymes. Fifteen hypothetical ORFs were differentially regulated. The hypothetical ORFs that are differentially regulated during interaction with human cells may represent genes required for infection and could be the focus of future work.

These experiments demonstrate that transcriptional profiling experiments can help classify the activities of many genes whose functions have previously been unknown.

4.5. Identifying Genomic Variation Using DNA Microarrays

DNA microarray hybridization analysis is increasingly being used to look at genomic variation in pathogens. An interesting offshoot of this technology is its use for rapidly analyzing strain differences among pathogenic bacteria such as *Mycobacterium tuberculosis*, the causative agent of tuberculosis. The vaccines developed against this organism were generated from the Bacille Calmette–Guerin (BCG) family of an attenuated strain of a related bacterium, *M. bovis*. Using microarray analysis, Behr et al. (1999) discovered that a region conserved in all virulent *M. tuberculosis* strains is missing in all *M. bovis* BCG vaccine strains. Eleven regions containing 91 genes from one or more virulent strains of *M. tuberculosis* are missing in *M. bovis*. In

addition, five regions are present in *M. bovis* and *M. tuberculosis* that are absent in some or all BCG vaccine strains. Thus, this technology may soon affect the development of vaccines for future use.

In a similar analysis of *E. coli* evolution, Ochman and Jones (2000) compared the 4,290 ORFs from the sequenced strain K12 MG1655 to four related strains, one closely related and three others more distantly related. The DNA from each ORF was spotted onto nylon membranes in duplicate and probed with labeled genomic DNA. Ochman and Jones found that a maximum of 3,782 ORFs were common to all strains. The most closely related strain (W3110) contained 65 kb of DNA not found in MG1655 and lacked 82 kb of DNA found in MG1655. The most distantly related strain (ECO37) contained more than 1,100 kb of DNA not found in MG1655 and lacked 392 kb of DNA found in MG1655. Ochman and Jones suggest that all differences among the five strains can be accounted for by 67 molecular events: 37 insertions and 30 deletions.

In addition to the *E. coli* and *Mycobacterium* analyses described above, strain differences have also been analyzed by DNA microarray analysis for *Helicobacter pylori* (Salama et al., 2000), *S. aureus* (Fitzgerald et al., 2001), *Streptococcus pneumoniae* (Hakenbeck et al., 2001), *Salmonella typhimurium* (Porwollik et al., 2002), *Pseudomonas aeruginosa* (Wolfgang et al., 2003), and *Streptococcus* serotype M18 group A strains (Smoot et al., 2002). These analyses have demonstrated that microarray technology can detect a wide variety of genomic rearrangements. The scope of genomic rearrangements suggests new ways of understanding pathogenic biology and evolution.

4.6. Problems with Microarray Technology

Despite the potential of microarrays, there is still a great deal of skepticism about the technology. This is due, in part, to problems inherent in the technology. One problem is the many different types of controls that are needed to ensure the biological relevance of any one experiment. Each control represents an independent variable, and these variables must be taken into consideration when both biological and process replications of each experiment are performed. Statistical analysis is also needed to ascertain the relevance of any individual measurement. As a consequence, it is still best to take the results produced by this procedure as preliminary. At present, microarray technology is best used to define candidate genes for a particular condition. These candidate genes then need to be tested individually.

5. Comparative Genomics

5.1. Pregenomics Taxonomy

Initially, bacteria were categorized the same way as all fauna. For example, a mammal is defined by bone structure, method of breeding, presence of hair, etc. To the early microbiologists, differences in bacterial physiology and

morphology, such as shape or the presence of an outer membrane (e.g., Gram-positive vs. Gram-negative), represented methods of distinguishing bacteria. Today, *Bergey's Manual of Determinative Bacteriology* (Holt et al., 1994) categorizes many thousands of bacteria based on such characteristics as colony morphology, growth requirements, the biochemical makeup of the organism, and the array of enzymes encoded by the organism. These categorizations are remarkable in their complexity and in their ability to accurately organize bacteria into groups. However, there are problems in some of these characterizations.

5.2. 16S rRNA-based Taxonomy

In the 1970s, Carl Woese and others, understanding the limitations of morphological and biochemical characterizations and realizing the potential of the new sequencing technologies, chose rRNA genes as a foundation for defining the relationships of bacteria. By sequencing and comparing the 16S rRNA genes from many bacteria, Woese and others started to understand that relationships of bacteria were more complex than had been thought (Woese et al., 1990). Since then, characterization of bacteria using 16S rRNA sequence data has enabled microbiologists to more accurately define the relationships of many bacteria. These comparisons have supported the endosymbiotic origins of mitochondria (from a common ancestor of the α-Proteobacteria) and the chloroplast (from the common ancestor of the Cyanobacteria). They have also codified the Archaea as a separate domain of organisms. In fact, this analysis has largely overturned the paradigm of animal classification of the last century. The Five Kingdoms (Animals, Plants, Fungi, Bacteria, and Protists) are now the Three Domains (Eubacteria, Archaea, and Eukaryota). Bacteria are also classified into groups depending on their 16S sequence. In addition to classifying *E. coli*, for example, as a Gram-negative rod, it can now also be defined as a member of the γ-Proteobacteria.

5.3. Horizontal Gene Transfer

"Whereas prokaryotic and eukaryotic evolution was once reconstructed from a single 16S ribosomal RNA (rRNA) gene, the analysis of complete genomes is beginning to yield a different picture of microbial evolution, one that is wrought with the lateral movement of genes across vast phylogenetic distances."

Jain et al. (2002)

In the late 1980s and early 1990s, as the sequences of more genes were completed, problems with phylogenetics based on 16S rRNA sequences appeared. For some prokaryotic organisms, the phylogenetic relationship of many of their proteins suggested a different evolutionary pattern than the phylogenetic relationship of their 16S rRNA sequence. These differences, or *incongruences*, could be explained by the movement of genes between distinct

species over evolutionary time. This movement of genes has been called *horizontal transfer*. This is distinguished from *vertical descent*, the acquisition of genes from an ancestor. Vertical descent is the most common form of evolutionary inheritance in eukaryotes.

The ubiquity of horizontal gene transfer was most dramatically shown when the genomes of two uropathogenic strains of *E. coli* O157:H7 were published (Hayashi et al., 2001; Perna et al., 2001). By comparing the genomes of these pathogenic strains with the genome of nonpathogenic *E. coli* K12, researchers hope to learn which genes enable a benign organism to become pathogenic. The significant conclusion of these studies is that chromosomes of highly related bacteria that occupy different niches share a common backbone that is interrupted by many variable regions. Perna and coworkers showed that one of the pathogenic isolates (EDL933) contains 1,387 genes that are not present in K12; conversely, 528 genes are present in K12 that are not present in EDL933. The two *E. coli* strains contain an *E. coli* backbone in which 88% of the genes common to the two genomes have greater than 70% identity, but the overall genomes differ by 25%. Most of these differences reside in regions termed *pathogenicity islands* (See Chapter 4). Comparison of guanine–cytosine (GC) content (see later) suggests that most events are due to recent horizontal events. Their putative protein sequences suggest that many of the genes unique to O157:H7 encode toxins. Figure 5 shows a model of the genome of *E. coli* O157:H7 strain EDL933 (Perna et al., 2001). Shown in the figure are regions that are distinct between EDL933 and another *E. coli* O157:H7 strain RIMD (Hayashi et al., 2001).

In another important study, the combination of comparative genomics and microarrays has recently provided compelling evidence to suggest that almost one quarter of the entire *S. typhimurium* genome may have been acquired by horizontal gene transfer (Porwollik and McClelland, 2003).

5.4. Methods for Detecting Horizontal Transfer

Currently, there are three recognized mechanisms for horizontal transfer in bacteria: transformation (in which cells take up free DNA); conjugation (transfer of plasmid DNA through the pilus apparatus); and transduction (transfer of DNA via phage). These processes are discussed in more detail in Chapter 2. Five methods have been used to determine horizontal transfer between genomes. These are GC content, phylogenetic analysis, synteny, codon usage, and genome signature (Jain et al., 2002).

Percentage of GC content of an organism is determined by counting the number of the G and C nucleotides in the genome and dividing by the total number of nucleotides. Organisms that have a high percentage of GC content have a bias toward either G or C in the third position of the codons. This bias is also reflected in the codon usage of the organism and is specific for the organism. This GC content is consistent throughout the genome, and thus

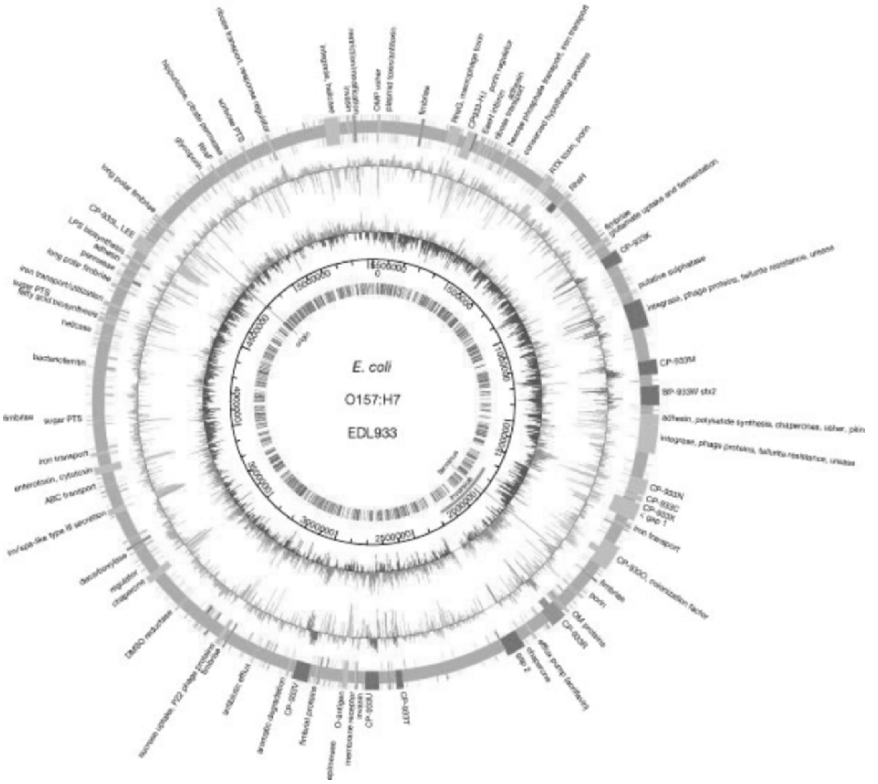


FIGURE 5. Circular genome map of EDL933 compared with MG1655. The first outermost circle shows the distribution of islands: shared co-linear backbone (blue); position of EDL933-specific sequences (O-islands) (red); MG1655-specific sequences (K-islands) (green); O-islands and K-islands at the same locations in the backbone (tan); hypervariable (purple). The second circle shows the G + C content calculated for each gene longer than 100 amino acids, plotted around the mean value for the whole genome, color-coded like the outer circle. The third circle shows the GC skew for third-codon position, calculated for each gene longer than 100 amino acids: positive values (lime); negative values (dark green). The fourth circle gives the scale in base pairs. The fifth circle shows the distribution of the highly skewed octamer Chi (GCTGGTGG), where two DNA strands are indicated (bright blue and purple). The origin and terminus of replication, the chromosomal inversion and the locations of the sequence gaps are indicated. (Created by Genvision from DNASTAR.) (See color insert.)

regions that do not match the GC content may be areas of DNA derived by horizontal transfer. This is currently the most-used tool for determining whether a region of a genome has been horizontally transferred.

Phylogenetic analysis attempts to distinguish the relationships among homologous genes and proteins derived from different organisms by analyzing and comparing specific sequences encoded by the organisms. This

method was first developed in the 1970s by Dayhoff et al., (1983). Phylogenetic analysis consists of two steps: in the first, an alignment is generated using a similar set of proteins or genes; in the second, differences among the sequences are determined at all residues. These differences can be given a mathematical representation that is displayed in the form of a tree. The likelihood of horizontal transfer can be implied by comparing the tree generated by specific protein sequences to that generated by the 16S ribosomal genes.

Synteny is the presence of homologous genes on two genomes in the same order on the chromosome. Syntenic regions that are interrupted by heterologous sequences likely are events of either intragenomic rearrangements or horizontal transfer. These two possibilities are distinguished by phylogenetic analysis of the interrupting sequences.

Codon usage is the analysis of the codons used in the genes of an organism. It is known that genes in a given genome have a bias in their choices among synonymous codons, and this bias is a reflection of the abundance of charged tRNAs in the cell. As a consequence, genes that have a codon bias that is dramatically different from the organism's codon bias may represent genes derived by horizontal transfer.

Genome signature is the relative abundance profile of di-, tri-, and tetranucleotides present in the genome. This method, developed by Karlin and colleagues (Karlin and Burge, 1995; Karlin et al., 1997), uses mathematical modeling to show that, at a resolution of 50 kb regions, these relative abundances can be viewed as characteristic to that genome as a result of selective pressure. Areas of the genome that do not match the genomic signature are viewed as regions of possible horizontal transfer.

6. Themes of Pathogenicity Determined from Genomic Analysis

6.1. Evolution Driven by Horizontal Gene Transfer

A major conclusion that has resulted from the study of bacterial genomes is that horizontal gene transfer is the predominant method for driving the evolution of pathogens into new niches. To date, most sequenced pathogens have DNA regions that contain genes required for virulence and pathogenicity when compared with closely related nonpathogenic genomes. These regions appear to have been horizontally transferred as determined by the percentage of GC content or codon usage. The likelihood of horizontal transfer becomes clearer as more genomes of the same species are obtained. There is a high correlation between altered GC content and possible horizontal gene transfer. Regions high in GC content and codon usage variation are also high in phage and insertion sequences as well as in pathogenicity factors.

6.2. Pathogenicity Islands

Many pathogenic organisms contain regions in their genomes called *pathogenicity islands*. These regions are so designated because they contain many virulence factors (see Chapter 4). The position of the islands in a given genome is relatively constant, but the actual genes vary dramatically. In addition, the types of virulence factors vary among different strains, isolates, and serovars. These differences are believed to encode the proteins determining host specificity and levels of virulence. Reid et al. (2000) showed clearly in *E. coli* that several virulence factors had been acquired in parallel. This variation was also seen in *S. aureus,* where methicillin resistance had been acquired multiple times by *S. aureus* clones (Fitzgerald et al., 2001; Kuroda et al., 2001).

6.3. Plasmids as Mobile Pathogenicity Islands

Large plasmids in some bacteria act as mobile pathogenicity islands. Many pathogens harbor plasmids that contain genes associated with pathogenicity, including *Agrobacterium tumefaciens* (a pathogen of plants), *Brucella melitensis* (a potential biological warfare pathogen that causes a recurring debilitating disease in humans), *Vibrio cholerae*, *Shigella* spp, and *S. typhimurium* (important causes of diarrheal disease in humans) (DelVecchio et al., 2002; Goodner et al., 2001; Heidelberg et al., 2000; McClelland et al., 2001; Wood et al., 2001). Such extrachromosomal DNA is probably quickly lost from a host strain when there is not strong and continuous selection. An interesting "twist" to this concept is the common soil bacterium *A. tumefaciens*, which has two conserved megaplasmids that are required for, and associated with, plant virulence. In addition, *Agrobacterium* has both a linear chromosome and a circular one. The DNA replication apparatus of the linear chromosome suggests that it actually began as a captured plasmid (Goodner et al., 2001). Thus this "linear plasmid" must have been extremely stable to have passed through all the changes necessary to have evolved from its original circular form (see Chapter 4).

6.4. Hypervariable Regions

Some regions in genomes are hypervariable (manifested by a high frequency of insertions and deletions); these regions often contain pathogenicity islands. This phenomenon often occurs in organisms such as *Listeria*, *E. coli*, *Staphylococcus*, and *Helicobacter*. In *H. pylori* and *Chlamydia trachomatis*, these regions of extreme variability are called *plasticity zones* (Alm et al., 1999; Kalman et al., 1999; Read et al., 2000; Salama et al., 2000; Shirai et al., 2000; Stephens et al., 1998; Tomb et al., 1997). These hypervariable regions appear to account for functions necessary for host specificity and for many of the mechanisms of pathogenicity.

6.5. *Reduced Horizontal Transfer in Intracellular Pathogens*

Obligate intracellular pathogens appear less likely to have genes acquired by horizontal transfer. The intracellular pathogens showing the fewest putative horizontal transfer events include *M. leprae*, *Rickettsia prowazakii*, and *C. trachomatis* (Andersson et al., 1998; Cole et al., 2000; Stephens et al., 1998). These organisms are obligate intracellular pathogens, or they have distinct lifestyles (such as very slow growth). One possible reason for the lower level of horizontally transferred genes is that the organisms are in contact with fewer other pathogens and thus may not be exposed to the high number of pathogenicity-causing genes in the genome space. Another possibility is that these genes give the organism no selective advantage and are thus not retained through selection. A third possibility is that these organisms are more recently derived and have not had the time to collect horizontally transferred genes. Whatever the mechanism, the evolution and selective pressure on these genomes are more affected by vertical descent than by horizontal transfer.

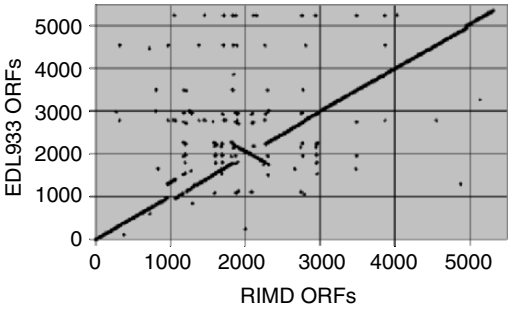
7. Genomic Rearrangements—Syntenic Maps Give a View of Vertical Descent

The most common way for most genes to be derived is by vertical descent. To give an example of conservation of genes by vertical descent and the use of comparative genomics, we have generated syntenic maps of five bacteria from the Enterobacteria (Figure 6), including three strains of *E. coli*—K12 (Blattner et al., 1997), O157:H7 EDL933 (Perna et al., 2001), and O157:H7 RIMD (Hayashi et al., 2001), *S. typhimurium* LT2 (McClelland et al., 2001), and *Yersinia pestis* KIM (Deng et al., 2002). This simple genomic analysis gives a visual representation of two methods used to examine the evolution of genomic architecture by vertical descent and intramolecular rearrangements within a genome over time. Intramolecular genomic rearrangements occur frequently and are detected by genomic comparisons such as these.

Genomes that are perfectly syntenic would generate a straight line on the 45-degree diagonal starting with position 1 at the lower left. We can easily see the two genomes that are most conserved and least conserved. The straightest line is generated by the comparison of the two *E. coli* O157:H7 strains, EDL933 and RIMD (Figure 6A). The least-straight line is generated by the comparison between the *E. coli* K12 and the *Y. pestis* genomes (Figure 6B). In fact, just a short analysis shows that RIMD is most closely related to EDL933 and that *E. coli* is most distantly related to *Y. pestis*. A comparison between the *E. coli* strains K12 and EDL933 (Figure 6C) shows that the line is not as straight as between the two pathogenic *E. coli* strains, which suggests that the two strains are not as similar as EDL933 and RIMD. The same is

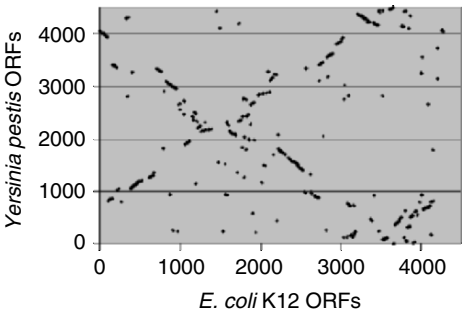
A

Synteny between two *E. coli* O157:H7 (EDL933 and RIMD)



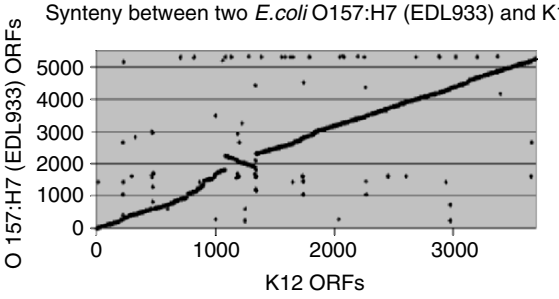
B

Synteny between *Yersinia pestis* KIM and *E.coli* K12



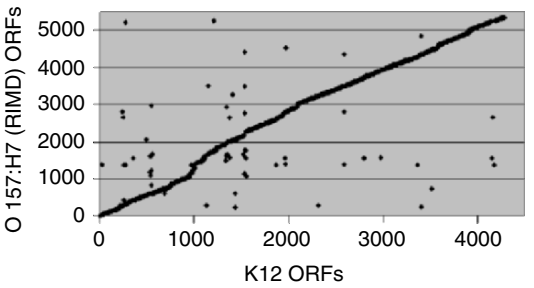
C

Synteny between two *E.coli* O157:H7 (EDL933) and K12



D

Synteny between two *E.coli* O157:H7 (RIMD) and K12



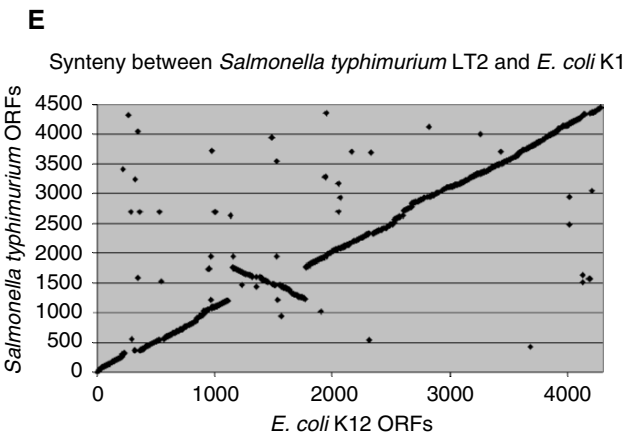


FIGURE 6. Syntenic maps can be used to analyze the evolutionary relationships among related genomes. Syntenic maps of five bacteria from the Enterobacteria: three strains of *E. coli*—K12 (Blattner et al., 1997), O157:H7 EDL933 (Perna et al., 2001), and O157:H7 RIMD (Hayashi et al., 2001)), *Salmonella typhimurium* LT2 (McClelland et al., 2001), and *Yersinia pestis* KIM (Deng et al., 2002). To determine the syntenic relationships between these organisms, genome plots were generated (Goodner et al., 2001). To generate these plots, a homology search using the BLAST algorithm of the predicted proteins of *E. coli* K12 was run against each of the other five genomes. The algorithm identifies the top hit for each protein in each genome. The relative chromosome location of each top hit protein in each genome was then compared. For example, aspartokinase is the first protein encoded next to the origin of replication in *E. coli* K12. If the top hit for *E.coli* O157:H7 EDL933 is aspartokinase and it is also encoded next to the origin of replication, a syntenic plot would place the genes encoding these two proteins at coordinates 1,1. The two genomes must have an identity of 75% over the length of the protein to appear on the graph. If the two genomes are perfectly syntenic, a diagonal line is generated running from the lower left corner to the upper right corner. Any inversion will appear as a diagonal line running at a 90-degree angle relative to the rest of the genome. Duplications are displayed as parallel lines off of the diagonal. Genomes that have the straightest lines represent those genomes that are less altered relative to one another. In this way, a simple analysis of genes derived by vertical descent can be generated. Any gene that has been lost and subsequently reacquired will most likely not occur in the same location in the genome.

also true for a comparison between K12 and RIMD (Figure 6D). The lines suggest that the differences between *E. coli* K12 and *S. typhimurium* (Figure 6E) are roughly the same as the differences between *E. coli* K12 and the pathogenic strains of *E. coli*, EDL933 and RIMD (Perna et al., 2001).

For a small section of this graph (centered around the ORF 2000 region), the line runs from the upper left to the lower right. This segment represents an inversion between the two genomes. There is also an inversion centered on ORF 1200 of the K12 genome (which is equivalent to the ORF 2000 region of the EDL933 genome). A comparison of either *E. coli* or *S. typhimurium*

with *Y. pestis* suggests that numerous inversion events have occurred during the evolution of the species. We can see by these graphs that even simple analyses of genomic data can generate new and interesting views of bacterial evolution.

8. Conclusions

This is the Golden Age of bacterial biology. The number of sequenced bacterial genomes and the accompanying papers describing their impact are rising dramatically. The first bacterial genome, *H. influenzae*, was completed in 1995; by the end of 2003, almost 130 genomes had been completed. It is clear that we are just beginning to understand the knowledge generated by these technologies. For example, we are beginning to understand several key features of the mechanisms of pathogenesis as well as the evolution of pathogenesis.

The fields of genomics, bioinformatics, and transcriptional profiling are all new and prone to errors generated by their very novelty. In the future, the data generated by these platforms will be more highly integrated and merged into systems biology. As bioinformatics technologies develop, scientists will more easily generate and manipulate these large data sets. By integrating biological data, such as protein content (*proteomics*) and intermediary metabolite content (*metabolomics*), with these technologies, we will better understand the value of such data and how to interpret them. In addition, we will have higher confidence in our annotation and be able to draw greater inferences on the biology of bacteria.

8.1. Genomic Space

Evidence shows that lines of *E. coli* have acquired the same virulence factors in parallel (Reid et al., 2000). Therefore, understanding the evolutionary forces that drive the selection of pathogens requires a thorough understanding of which genes were derived from an ancestor, vertical descent, as opposed to horizontal transfer. An intriguing idea proposed by Dobrint and Hacker (2001) is called "flexible gene content." This theory posits that all genomes have a core of genes that are required for living in all environments. These genomes are constantly expanding and contracting due to horizontal transfer and deletion and mutation. These new genes, which allow an organism to invade and survive in a new environment, provide a "flexible" gene set that can be passed on to succeeding generations.

The genes necessary for pathogenicity reside in *genomic space*, not in any particular organism. The concept of genomic space is abstract and refers to the sum of all genes found in all of the organisms in an environment that are able to provide genetic material to other organism via hortizontal trans-

fer. For pathogenicity, that genetic material comprises all of the genes necessary to enable an organism to enter a host, evade or disable its self-defense machinery, and cause disease. Thus, the pathogenicity of the *genomic space* comes from the genes that allow the organism to infect and negatively impact the health of a host. This *genomic space* contains many genes required for pathogenicity that provide either host or tissue specificity. These genes are constantly being shuffled around from one genome to the next, sometimes as operons (see Lawrence and Roth's selfish operon theory (1996)) and sometimes as single genes. The mechanisms of transfer are mediated by phage, plasmids, or competency of the bacterial host organism. In some cases, the organism has competence machinery that is designed to import DNA from external sources (e.g., *Neisseria* and *Haemophilus*). In other cases, prophage or phage fragments provide the genes necessary for this transfer (e.g., *Vibrio*). The constant shuffling of genes provides the genetic resources for a pathogen to continue to survive in a hostile environment.

Questions to Consider

1. What is the standard genome coverage used to sequence an organism?

6–10× coverage. Closing the genome requires more reads, but finishing requires a more focused effort.

2. How can an ORF be incorrectly annotated?

Through improper gene models, improper algorithm implementation, and human or computer errors.

3. In what ways can horizontal gene transfer be determined?

There are five basic methods: GC content, phylogenetic analysis, synteny, codon usage, and genome signature. These methods are taken together to determine if a particular gene or region has been derived by vertical descent or horizontal transfer.

4. What developments were necessary for the implementation of genomic analyses?

The development of improved sequencing machinery, high-speed computers, and algorithms that could handle large amounts of data.

5. What kinds of information can be generated by transcriptional profiling?

A view of the transcriptional output of the entire genome at one time point under one condition. Transcriptional profiling has also been used to analyze gene content of closely related organisms.

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Chapter 4

Pathogenicity Islands and Bacterial Virulence

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Historical Landmarks

1990 Definition of unstable chromosomal regions with virulence genes as "pathogenicity islands" (PAIs) in uropathogenic *Escherichia coli* by Hacker et al. (1990).

1995 Observation that invasion genes of S*almonella enterica* are clustered within a 40 kb region of the chromosome (Mills et al., 1995).

1996 Identification of a PAI of systemic virulence in *S. enterica* and definition of *Salmonella* Pathogenicity Island (SPI) (Shea et al., 1996).

1997 Experimental transfer of the PAI Locus of Enterocyte Effacement (LEE) of enteropathogenic *E. coli* to a nonpathogenic *E. coli* strain (McDaniel and Kaper, 1997).

1999 Identification of a PAI of bacteriophage origin in *Vibrio cholerae* (Karaolis et al., 1999).

1. Introduction

Bacteria have a remarkable ability to adapt to new environments and changing growth conditions. This is also the case for pathogenic bacteria, as they have developed various strategies to invade and colonize plants, animals, and humans. Complex virulence traits can be found in related forms in different pathogens, indicating that the corresponding genetic information has been efficiently distributed during bacterial evolution. It has been observed that many of these virulence genes are clustered within distinct regions of the bacterial chromosome, which are absent from nonpathogenic relatives. These regions were termed "pathogenicity islands" (PAIs) and have several unique features. While PAIs were initially discovered as chromosomal regions, they are also found carried on virulence plasmids. PAIs have been distributed between bacteria by horizontal gene transfer, a mechanism that promotes the rapid spread of virulence factors between various groups of bacteria.

2. How Did Bacteria "Learn" to Infect and Colonize Host Organisms?

Related virulence factors can be found in different pathogens. Horizontal gene transfer contributed to the distribution of corresponding genes between bacterial species. Certain virulence functions of pathogenic bacteria are surprisingly complex. Good examples are systems for protein translocation that are found in Gram-negative bacteria. Such type III and type IV secretion systems (T3SS and T4SS) require the function of 20–30 genes to assemble

complex molecular machines, which are able to inject protein toxins directly into eukaryotic target cells (see Chapter 9; also see Thanassi and Hultgren, 2000 for an overview on bacteria protein secretion systems). In addition to these machines for protein secretion, specific regulators are present that control expression of the systems in a specific phase of pathogenesis (see Chapter 13). Obviously, the evolution of such complex systems would require an extremely long period of time if the entire system would have to be built de novo. A common theme in evolution is the modification and adaptation of a functional system. In eukaryotic organisms, this evolutionary step is often realized by duplication of genes, and the subsequent adaptation of the gene copies to specific functions. Although gene duplication followed by modification is also an evolutionary mechanism in bacteria, a more important means of evolution is sharing of functions by different species.

Analyses of bacterial pathogenesis resulted in the frequent observation that virulence factors can evolve very rapidly. However, similar observations have been made for environmental bacteria, indicating that adaptation to new habitats or to changing environmental conditions is a general ability of bacteria. This ability requires a specific mechanism by which genetic information is distributed between bacteria, which we refer to as "horizontal gene transfer."

2.1. Horizontal Gene Transfer

Normally, genetic information is passed from one generation to the next generation of individuals. This process is referred to as vertical gene transfer and is the basic mechanism of maintenance of genetic information in all living organisms. In addition, various organisms have developed an additional mechanism to transfer genetic information between individuals. Here, genetic information is shared between individuals of one generation, or even more, between the members of a population. This mechanism is termed horizontal gene transfer.

One obvious difference between vertical and horizontal gene transfer is the available expression of the genetic information. Vertical gene transfer requires the formation of a new generation of individuals that will express the inherited genetic information. In contrast, genetic information obtained by horizontal gene transfer is available immediately to the receiving individual and does not rely on the formation of a new generation.

3. Features of Pathogenicity Islands

PAIs have several characteristic features. However, not all PAIs fulfill all criteria. What are the common features of a PAI? A strict definition of a PAI is not possible, since the genetic elements referred to as PAI are very different in their structure and function. Also, the term PAI has become very popular

and diverse genetic elements have been designated as PAIs. However, there are common features that are found in most PAIs (see Box 1, and Figure 1 for schematic representation). It is important to note that most PAIs do not meet all of these criteria. The characteristics of PAIs listed below will also be demonstrated with examples from important pathogens later in this chapter.

3.1. Pathogenicity Islands Harbor Virulence Genes

One or several genes encoding virulence functions are located within PAIs. Examples of virulence factors encoded by PAIs include, but are not limited to, adhesins, toxins, invasins, iron uptake systems, and protein secretion systems. Genetic analysis of many pathogenic bacteria has shown that several virulence functions are clustered in a distinct region of the chromosome. These virulence gene clusters have several characteristic features and are called "Pathogenicity Islands." This designation may reflect that clusters of virulence genes appear as islands in a "sea of genes" not related to virulence functions. PAIs have been initially described for uropathogenic strains of *Escherichia coli* (Hacker et al., 1990), but since then, similar structures have been observed in nearly all groups of bacterial pathogens (Table 1).

3.2. Pathogenicity Islands Form Insertions in the Genome of Bacteria

PAIs are large insertions of horizontally acquired DNA in pathogenic bacteria. These insertions are absent in nonpathogenic relatives or avirulent

Box 1. Common features of pathogenicity islands

- Presence of one or more virulence genes:
  - Most PAIs encode complex virulence traits that require the function of a large number of genes. There are also clusters of virulence genes with different functions.
- Different base composition compared to the genome:
  - Each bacterial species is characterized by a specific base composition of the chromosomal DNA. PAIs contain differences in base composition from the host genome.
- Genetic instability:
  - Deletions of PAIs have been observed in several cases, especially if genes involved in mobilization of DNA are present. However, other PAIs appear to be genetically stable.
- PAIs form large insertions absent in nonpathogenic strains or related species, and are distinct genetic entities:
  - A PAI is only present in the pathogenic strain or species but not in an environmental or commensal strain or species.
  - PAIs have characteristic start and end points and a defined size.
  - The size of a PAI can vary within a range of 10–200 kb.
- Characteristic insertion point:
  - tRNA genes often serve as target region for integration.

It is important to note that most PAIs do not meet all these criteria.

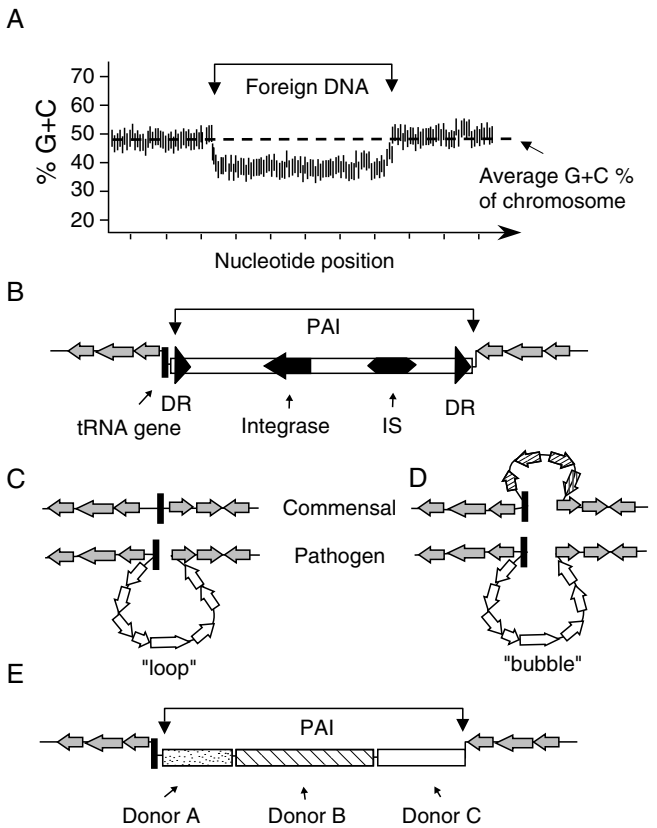

FIGURE 1. Specific features of PAIs are indicated in the context of a hypothetical PAI. (A) A region of the chromosome with a base composition different from the core chromosome indicates a horizontally acquired DNA element. Many PAIs show such typical differences in base composition. The percentage of G+C within a window of nucleotides can be lower (as shown) or higher than the average G+C content (red line) of the core chromosome. (B) Characteristic genes that function as target structures for integration of foreign DNA or DNA mobility are associated with PAIs. Many PAIs are flanked by genes encoding tRNAs (green bar). Direct repeat (DR), short identical or nearly identical sequence repetitions, can be found at the ends flanking PAIs. Insertion sequences (IS), integrases or phage genes within a PAI, are further indications for the previous acquisition by horizontal gene transfer and present mobility of the locus. (C) Comparison of the genome structure of pathogenic and nonpathogenic or commensal bacteria indicate distinct insertions of large genetic elements between conserved genes present in both organisms (gray gene icons). In a hypothetical alignment, the insertion of a PAI (yellow gene icons) will form a loop-like structure. (D) The points of insertion of a PAI may also serve as point of insertion of genetic elements in nonpathogenic bacteria. For example, a PAI and a metabolic island (hatched gene icons) insert adjacent to the same tRNA gene. This will result in a bubble-like structure. (E) Analysis of the structure of certain PAIs indicates a mosaic structure. These PAIs are composed of several elements from different donors that have inserted at the same locus.

TABLE 1. Characteristics of PAIs of important pathogens

Species/strain	PAI name	Size (kb)	tRNA[a]	Stablility[b]	Mobility	Virulence functions
Dichelobacter nodosus	*vap*	12	*serV*	n.d.	phage, DR	Vap antigens
D. nodosus	*vrl*	27	—	n.d.	n.d.	Vrl antigens
Escherichia coli 536 UPEC	PAI I	77	*selC*	low	DR, *int*	Hemolysin
E. coli 536 UPEC	PAI II	102	*leuX*	low	DR, *int*	Hemolysin, P-fimbriae
E. coli 536 UPEC	PAI III	68	*thrW*	low	DR, *int*	S-fimbriae
E. coli 536 UPEC	PAI IV	30	*asnT*	low	*int*	Siderophore synthesis, iron uptake, see HPI
E. coli EHEC	LEE	43	*selC*	high	phage	Translocation of Tir, T3SS
E. coli EPEC	LEE	35	*selC*	high	no	Translocation of Tir, T3SS
Helicobacter pylori	*cag*	40	—	low	DR	Translocation of CagA, T4SS
Listeria monocytogenes	*plf vir*	9	—	n.d.	n.d.	Phospholipase, listeriolysin, etc.
Salmonella enterica	SPI-1	40	—	mid	no	Invasion and enteropatho-genesis, T3SS
S. enterica	SPI-2	40	*valV*	high	no	Intracellular proliferation, T3SS
S. enterica	SPI-3	17	*selC*	n.d.	n.d.	Intracellular proliferation, Mg^{2+} uptake
S. enterica	SPI-4	25	?	n.d.	n.d.	Unknown
S. enterica	SPI-5	7	*serT*	n.d.	n.d.	Effector proteins for SPI-1 and SPI-2
S. enterica sv. Typhi	"major PAI"	136	*pheU*	n.d.	n.d.	Vi antigen, SPI1 effector, type IV pili
Shigella flexneri	SHI-1	25	—	n.d.	n.d.	Enterotoxin, protease
S. flexneri	SHI-2	23.8	*selC*	n.d.	n.d.	Aerobactin, immunity to colicin V, etc.
S. flexneri	SHI-O	10.6	—	n.d.	phage	LPS biosynthesis
S. flexneri	SRL	66.3	*serX*	low	phage	Resistance to various antibiotics
Staphylococcus aureus	SCC*mec*	36–53[c]	—	n.d.	DR, IS, Tn	Methicillin resistance, other antibiotics
S. aureus	*v*Saα	25–40[c]	—	n.d.	DR, *tnp*	Enterotoxins

TABLE 1. Characteristics of PAIs of important pathogens—Cont'd

Species/strain	PAI name	Size (kb)	tRNA[a]	Stablility[b]	Mobility	Virulence functions
S. aureus	*v*Saβ	30–40[c]	—	n.d.	DR, *tnp*	Superantigens, enterotoxins
Vibrio cholerae	VPI	39.5	—	low	phage, DR	TCP-adhesin, regulator
Yersinia enterocolitica	HPI	43	*asnT*	mid	DR	Siderophore synthesis and uptake
Y. pestis	HPI/*pgm*	102	*asnT*	low	DR, IS	Sidereophore, pigmentation
Y. pseudotuberculosis	HPI	36	*asnT*, *U*, *W*	low	DR	Siderophore

[a] Indicates the presence of gene loci for tRNA that are adjacent to the PAI.
[b] Indicates whether the PAI is frequently deleted or stably inserted into the genome.
[c] Indicates variations between different strains.
Abbreviations: DR, direct repeat; IS, insertion sequence; n.d., not determined; T3SS, type III secretion system; T4SS, type IV secretion system; Tn, transposon; TSST, toxic shock syndrome toxin.

strains. Following detailed analysis of numerous gene loci containing clusters of virulence genes in a variety of pathogenic bacteria, it became obvious that these sequences were absent in their nonpathogenic counterparts. Comparison of genomes of nonpathogenic bacteria and pathogenic relatives can reveal differences in the organization of their respective genomes. Such differences may appear as loop-like structures, if a PAI has been inserted between two consecutive genes in the chromosome. This structural feature of PAIs is shown schematically in Figure 1A and B. There are also examples for the insertion of specific elements in the pathogenic and nonpathogenic species, resulting in a "bubble" when the two genomes are aligned. Many PAIs have been acquired in a single step of horizontal gene transfer; however, there are also indications that PAIs are composed of two or more elements that have been sequentially acquired by insertions at the same targeting site (Figure 1E). In such composite PAIs, the structural characteristics may vary in parts of different origin.

3.3. Base Composition of Pathogenicity Islands

Many PAIs have a base composition that is different from the characteristic base composition of the core chromosome. The base composition of bacterial DNA is a specific characteristic of each individual species. The average base composition of DNA can range from 25% guanosine and cytidine (G+C) to 75% G+C. Most pathogenic bacterial species have G+C contents between 40% and 60%. The reasons for the variation are not known, but the conservation of a genus- or species-specific base composition is a remarkable

feature of bacteria. The base composition of PAI and other horizontally acquired elements often diverges from the average base composition of the main chromosome. Compared to the average base composition, a higher or lower G+C content can be found for many PAIs (see Figure 1A). It is considered that the horizontally acquired PAI still has a base composition similar to that of the donor species. On the other hand, it was also observed that the base composition of horizontally acquired DNA will ameliorate to the base composition of the recipient's genome during evolution. Thus, it is difficult to explain why "ancient" PAIs still show a different base composition. Further factors such as DNA topology or specific codon usage of the virulence genes in PAIs may also account for the maintenance of the divergent base composition.

3.4. Genetic Instability of Pathogenicity Islands

Many PAIs are genetically unstable. The same mechanisms that promoted the horizontal acquisition and integration into the chromosome are still active and can lead to the loss of the PAI. Virulence functions encoded by certain PAIs are lost with a frequency that is higher than the normal rate of mutation. Genetic analyses showed that such mutations are not caused by defects in individual virulence genes within the PAI, but rather by loss of large portions of a PAI, or even the entire PAI. These mutations can be observed during cultivation of pathogens in vitro, and also in isolates obtained from infected individuals, e.g., during persistent infections. These observations indicate that some PAIs have an intrinsic genetic instability. The same genetic mechanisms allowing the distribution of PAIs by horizontal gene transfer also determine their genetic instability. Several characteristic elements have been identified that contribute to mobilization as well as to instability. Some PAIs contain genes that are also found in bacteriophages. Lysogenic bacteriophages possess enzymes that mediate the integration of the phage genome into the genome of the host bacteria, as well as the excision, in order to enter a lytic cycle. Such genes are still functional in certain PAIs and the encoded proteins can mediate the excision of the PAI and its loss. The role of bacteriophages in transfer of PAIs will be described in Section 5.2.

Other PAIs contain genes that are similar to integrase and resolvase genes of transposons. These mobile genetic elements can change their location within the chromosome, but transposons can also "jump" from a chromosomal location into a plasmid and vice versa.

Direct repeats (DR) are further characteristic elements frequently observed at the flanking regions of PAIs. DR are DNA elements of typically 16–20 bp with a perfect or nearly perfect sequence repetition. Such sequences could have served as recognition sites for the integration of bacteriophages, and the integration resulted in the duplication of the sequence. DR also act as recognition sequences for enzymes involved in excision of mobile genetic elements, thus contributing to the instability of a PAI flanked by DR.

The loss of a PAI is probably promoted by the same mechanisms that contribute to the loss of resistance plasmids in the absence of selective pressure. Loss of a PAI results in a reduction in genome size leading to a reduced generation time that is of advantage in competition with other microbes.

Genetic instability of a PAI is a reflection of recent acquisition of this element, as the longer the element has been in the genome, the more stable it becomes. In PAIs that have become indispensable for the recipient, genes that are related to mobilization and instability become nonfunctional and are deleted. Thereby, the PAI becomes a part of the core genome. Good examples for stable PAIs are SPI-1 and SPI-2 of S*almonella enterica* that lack genes contributing to mobility and belong to the normal gene set of this pathogen.

3.5. Pathogenicity Islands and Genes for tRNAs

The insertion of PAIs into the chromosome are often located adjacent to genes encoding transfer RNAs (tRNAs), indicating that these genes represent "anchor points" that are similar in various bacteria. A remarkable observation is the frequent association of PAIs with gene loci encoding tRNAs. This observation gave rise to the hypothesis that tRNA genes serve as anchor points for insertion of foreign DNA that has been acquired by horizontal gene transfer. The frequent insertion may be explained by the observation that genes encoding tRNAs are highly conserved between various bacterial species. After acquisition by horizontal gene transfer, a DNA fragment that contains a tRNA gene can insert into the recipient's genome by recombination between the tRNA genes. The second observation is that certain bacteriophages use tRNA genes as specific insertion points in the host genome. Taken together, tRNA genes may represent specific anchor points for the integration of foreign DNA.

4. Identification of New Pathogenicity Islands

The specific characteristics of PAIs allow the application of *in silico* and experimental approaches for the identification of new PAIs. The knowledge of the common characteristics of PAIs will also enable investigators to identify new PAIs or PAIs in new pathogens (see Box 2). If the genome sequence of the pathogen is available, it is possible to scan for regions that have a different base composition (i.e., G+C content) from the rest of the genome. Since the base composition of bacterial DNA is a remarkably stable feature of each individual species, DNA sequences that differ in G+C content from the rest of the genome can be used to detect PAIs. This can be easily done by a computer program that plots the base composition within a given window of nucleotides. Another option is the analysis of potential sites of insertion of foreign elements. As many PAIs are inserted at tRNA genes, it can be of interest to examine the vicinity of tRNA genes for the presence of pathogen-specific insertions.

Box 2. How to identify new pathogenicity islands

Using the criteria for defining a PAI, one can screen bacterial genomes for the presence of PAIs.

- Using genetic screens:
  - Identification, cloning, and characterization of genomic elements that are present in the pathogen but absent in nonpathogenic relatives.
  - Genetic screens for unstable regions of the genome.
- If genome sequence data are available:
  - Screening for regions that are absent in a nonpathogenic relative.
  - Screening for regions with aberrant base composition of DNA.
  - Analyzing the vicinity of genes encoding tRNA.

Without the genome sequence, it is possible to clone and identify pathogen-specific genomic elements. The principle of subtractive DNA hybridization can be used for this purpose. Genomic DNA of a nonpathogenic species or strain is fragmented and immobilized on a matrix. Genomic DNA of the pathogenic species or strain is also fragmented and hybridized to the matrix. All DNA fragments common to both species are identified for DNA hybrids and are subtracted. Only those fragments that are specific to the pathogenic species will not form DNA hybrids and can be isolated subsequently. These fragments are then further characterized by subcloning into vectors or PCR amplification after ligation to universal adapters.

Another method is based on the instability of PAIs. A marker for negative selection is integrated into the bacterial chromosome. If selective pressure is applied, only those mutant clones will survive that have eliminated the selection marker or the region containing the marker. This approach thus allows positive selection for the rare events of the deletion of a PAI.

In general, identification of PAIs is possible if pathogenic and nonpathogenic strains or species are closely related. More distantly related species often have different genome organizations, thus making a comparison more difficult.

5. Evolution and Transfer of Pathogenicity Islands

Horizontal gene transfer can explain the observation that important virulence factors are present in very similar forms in different bacteria. But how are PAIs transferred between bacteria? Different scenarios can be developed to explain the transfer.

5.1. Transformation and Pathogenicity Islands (see also Chapter 3)

Foreign DNA can be taken up by transformation. Certain bacteria are capable of natural transformation. During certain phases of growth, transport systems are expressed that allow the uptake of free DNA from the environment.

Although the majority of this foreign DNA will be degraded, some fragments that harbor "useful" genes will be integrated into the genome of the recipient and maintained as a PAI. It appears possible that this mechanism allows uptake of DNA from distantly related species that will be maintained if the selective pressure selects for the newly acquired features.

5.2. Bacteriophages and Pathogenicity Islands

Bacteriophages have a high potential in transferring genetic material between bacterial hosts. Bacteriophages have been isolated from virtually all bacterial species; even obligate intracellular pathogens such as *Chlamydia* spp. have specific phages (Hsia et al., 2000). Bacteriophages are able to transfer bacterial virulence genes as passengers in their genomes. It may appear surprising that bacteriophages carry bacterial virulence genes in their genomes, but this fact may be explained by the coevolution between predator and prey. The occasional transfer of virulence genes by phages will allow the recipient bacteria to colonize new habitats, such as new host organisms or specific anatomic sites. This extension will also allow a more efficient spread of the bacteriophages. Therefore, the transfer of bacterial virulence genes as "passengers" in the viral genome can also be an evolutionary benefit for the bacteriophage. An exciting example for the contribution of bacteriophages is found in the rather recent evolution of virulence factors in *Vibrio cholerae* (see Box 3).

Many PAIs are too large to explain their transfer as passengers in bacteriophage genomes. For example, T3SS or T4SS encoded by PAIs comprise 25–40 kb DNA, which is almost equivalent to the total genome size of a bacteriophage. Here, other mechanisms are conceivable. Certain bacteriophages are capable of generalized transduction. Normally, for the replication of the phage within the host bacterium, copies of phage genome are packaged into phages heads. During replication, the host DNA is fragmented. Occasionally, the enzymes involved in packaging the phage genome make mistakes and place a fragment of the host genome into the phage head. The resulting particles are still able to infect new host bacteria, but instead of the phage genome, now a fragment of the bacterial DNA will be transduced. Given sufficient sequence similarity, recombination may occur and the transduced fragment is integrated into the genome of the new host.

5.3. Pathogenicity Islands and Virulence Plasmids

Similar clusters of virulence genes are present in PAIs and on virulence plasmids, indicating that episomal and chromosomal locations are possible for the same gene cluster. It was observed that certain clusters of virulence genes are present on PAIs in some pathogens, and also on virulence plasmids in other bacteria. The T3SS required for infection of epithelial cells by *Shigella* spp. is encoded by *mxi/spa* genes located on a virulence plasmid, whereas a related gene cluster that is required for the invasiveness of *S. enterica* serovar

Box 3. How to become a global player: *Vibrio cholerae*, pathogenicity island, and the phage conspiracy

V. cholerae is an important gastrointestinal pathogen that causes several pandemics, i.e., a worldwide spread of the disease with a high number of, often fatal, cases. The molecular analysis of events leading to the emergence of new *V. cholerae* strains that caused the recent cholera pandemic gives a remarkable example for the acquisition of PAIs, the vectors that are involved, and the effects on bacterial virulence. The major virulence factor of *V. cholerae* is the cholera toxin, and the effect of the cholera toxin on intestinal cells results in massive watery diarrhea. Although many other factors contribute to colonization of the gut by *V. cholerae*, simply speaking, *V. cholerae* strains harboring the cholera toxin genes (*ctx*) are virulent while strains without *ctx* are nonpathogenic.

During the last cholera pandemic, a novel virulent strain appeared termed "ElTor". Surprisingly, this strain belongs to a known serotype that was not previously associated with cholera pandemics and was lacking *ctx*. Somehow, the nontoxigenic strain converted into a highly pathogenic strain. In 1996, Waldor and Mekalanos (1996) identified the responsible element, a bacteriophage that carried *ctx* within its genome. This phage can infect strains of *V. cholerae* and, after infection, the phage genome can integrate into the genome of *V. cholerae*, a process termed lysogenic conversion. Lysogenic conversion results in new toxigenic strains, and the integrated phage with the toxin gene can be considered as a precursor of a PAI.

However, CTXΦ cannot infect all *V. cholerae* strains to cause lysogenic conversion. A further clue came from the analysis of the receptor for CTXϕ. The infection requires the presence of a pilus, which serves as receptor for CTXΦ. The genetic information of the *tcp* pilus assembly is located on a PAI and characterization of the *tcp* PAI revealed that this locus has several characteristics of other bacteriophages (Karaolis et al., 1999). It is now clear that the *tcp* pili are in fact structural elements of the phage.

A likely scenario for the evolution of new toxigenic strains of *V. cholerae* is as follows: The *tcp* phage infects a nontoxigenic *V. cholerae* strain and integrates into the chromosome. The expression of *tcp* allowes the infection with CTXΦ, and the integration into the chromosome results in a toxigenic strain. If these events occur in a serotype of *V. cholerae* that has not been frequently associated with human infections, the newly evolved strain can rapidly infect many individuals, since immunity to this serotype is absent in the population.

Typhimurium is located in *Salmonella* Pathogenicity Island 1 (SPI-1) on the organism's chromosome. Plasmids can be easily transferred between bacteria and replicate autonomously from the bacterial chromosome. Under certain conditions, plasmids may also integrate into the chromosome. In this regard, it is interesting to note that during the course of its evolution, the plant pathogen, *Agrobacterium tumefaciens*, appears to have incorporated a virulence plasmid into its genome (see Chapter 1). The virulence plasmids could therefore be considered as another means for transfer of PAIs between bacteria.

6. Paradigms of Pathogenicity Islands and Their Role in Bacterial Pathogenesis

In this section, the specific characteristics of selected PAIs and their roles in pathogenesis are described. Further PAIs are listed in Table 1. For more comprehensive descriptions of PAIs in various bacterial pathogens, see recent

reviews (Hacker and Carniel, 2001; Hacker and Kaper, 2000) or books on PAI (Hacker and Kaper, 2002a, b; Kaper and Hacker, 1999).

6.1. Pathogenicity Islands of Pathogenic Escherichia coli

PAIs were first identified in uropathogenic strains of *E. coli* as unstable regions of the chromosome. A variety of PAIs were identified in various pathogenic *E. coli*. The Gram-negative, rod-shaped species *E. coli* is a commensal in the human intestinal flora, but there are also a variety of pathogenic strains that are associated with intestinal infections, infections of the urinary tract, or septicemia. PAIs were initially identified in uropathogenic strains of *E. coli* (Hacker et al., 1990). Here, large genomic insertions were found that were absent in nonpathogenic strains of *E. coli*. Sequence analysis of these PAIs identified genes encoding hemolysins and adhesins, and also genes without an obvious contribution to virulence. A further feature of the PAIs in uropathogenic *E. coli* (UPEC) is the instability of the loci, as these elements were frequently lost. In enteropathogenic *E. coli* (EPEC), a PAI with a different function is present. This PAI is termed "Locus of Enterocyte Effacement" (LEE) and harbors the genes for a T3SS. The main function of this system appears to be the translocation of Tir, a protein that is phosphorylated by the host cell after translocation and inserted in the cytoplasmic membrane. Tir now serves as a receptor for the bacterial adhesin intemin. Intemin binding to Tir results in attachment and formation of pedestal-like protrusions of the host cell membrane. Interestingly, the transfer of LEE to nonpathogenic strains of *E. coli* is sufficient to transfer this virulence function (McDaniel and Kaper, 1997). This is an example of a PAI that contains all the genes necessary for the specific virulence function. Analyses of various pathotypes of *E. coli* indicated that LEE could be inserted at various chromosomal locations. In EPEC, LEE is inserted at the $tRNA^{selC}$ gene at 82 centisomes of the chromosome. The corresponding tRNA gene in other pathogens has served for insertion of PAI with functions different from that of LEE, such as SPI-3 of *S. enterica* or SHI-2 of *Shigella flexneri* (Blanc-Potard and Groisman, 1997; Moss et al., 1999).

6.2. The cag *Pathogenicity Island of* Helicobacter pylori

The *cag* PAI encodes a T4SS that mediates translocation of the cytotoxin CagA. *H. pylori* is a Gram-negative, spiral-shaped bacterium that is able to colonize the gastric mucosa, a body site that has long been considered as too hostile for bacterial life. Infections with *H. pylori* are frequent and often result in persisting colonization. This colonization can lead to the pathogenesis of gastric or duodenal ulcers, but certain forms of stomach cancer, such as mucosal-associated lymphoid tissue (MALT) lymphoma, are also associated with the infection. The search for virulence genes of *H. pylori* identified a PAI. The locus was termed *cag* PAI after the most prominent virulence factor, the translocated cytotoxin CagA that is encoded within the *cag* PAI (see Fig. 2a).

The remaining genes in the *cag* PAI encode another system for contact-dependent protein translocation. This T4SS of *H. pylori* is related to the *vir* system of *A. tumefaciens* that mediates the translocation of DNA–protein complexes into plants cells, inducing tumor formation in infected plants (Winans et al., 1996).

A strong correlation between the function of the *cag* PAI and the severity of *H. pylori* infection has been observed. The full pathogenic potential is found in type I strains that harbor a fully functional *cag* PAI, but infections with type II strains lacking the PAI are usually far less severe. Interestingly, there is also a continuum between type I and type II strains that correspond to the pathogenic potential of the strains (Covacci et al., 1997).

6.3. *The High Pathogenicity Island of* Yersinia *spp.*

The High Pathogenicity Island of *Yersinia* spp. encodes a siderophore and iron uptake system. This island is still mobile and can be found in other pathogenic enterobacteria. The genus *Yersinia* comprises three pathogenic species: *Y. pestis*, the causative agent of plague, and *Y. enterocolitica* and *Y. pseudotuberculosis*, food-borne pathogens causing gastrointestinal infections. In addition to the virulence plasmid that encodes major virulence factors, *Y. pestis* and highly virulent strains of *Y. enterocolitica* and *Y. pseudotuberculosis* also possess a PAI termed "High Pathogenicity Island" (HPI) (see Carniel, 2001 for a review). The main function encoded by HPI genes is a high-affinity iron uptake system (see Fig. 2c). One of the most limiting factors for bacterial colonization of a mammalian host is the availability of iron, and pathogens have evolved a variety of strategies to access the iron of the host organism. Genes within HPI encode the enzymes for the biosynthesis of yersiniabactin, a siderophore that can bind iron with a very high affinity. Additional genes encode a membrane-bound transport system for the uptake of iron-loaded yersiniabactin, as well as regulators of gene expression.

The HPI is characterized by genetic instability and a frequency of loss of 2×10^{-3} in *Y. pestis* to 10^{-4} in *Y. pseudotuberculosis* was reported (Carniel, 2001). A large number of DR and elements related to mobile DNA such as integrases and insertion sequences (IS) were detected within HPI, and these elements appear to contribute to the instability of the locus, as well as to the spread to other bacterial species. PAIs that are closely related to the HPI of *Yersinia* spp. are also present in other Gram-negative pathogens, and most frequently in pathogenic strains of *E. coli* (septicemic, uropathogenic, and enteropathogenic *E. coli*).

6.4. *Pathogenicity Islands in* Salmonella *spp.*

A large number of PAIs in *S. enterica* serovar Typhimurium are known. Two of these PAIs encode T3SS with functions in invasion of host cells, intracellular survival, and replication. *S. enterica* is an invasive, facultative

intracellular pathogen that is responsible for diseases ranging from localized self-limiting diarrhea to typhoid fever, a life-threatening systemic infection. Several PAIs have been identified in *S. enterica* serovar Typhimurium that are referred to as SPI. SPI-1 harbors genes encoding a T3SS that is involved in invasion into eukaryotic host cells. The genes in SPI-1 are very similar to the genes on the virulence plasmid of *Shigella* spp. that are also required for invasion. SPI-2 encodes a further T3SS that is required for the intracellular phenotype, i.e., the survival and replication in eukaryotic host cells (see Fig. 2b). SPI-1 and SPI-2 have different evolutionary origins and do not appear to be the result of duplication. SPI-3 harbors genes for a high-affinity magnesium uptake system that is required for the intracellular phenotype. This PAI is inserted at the *selC* tRNA gene, a locus that also serves as the integration point of a variety of PAI in other pathogens (Blanc-Potard and Groisman, 1997; McDaniel and Kaper, 1997; Moss et al., 1999). The role in virulence of SPI-4 has not been fully elucidated. Finally, SPI-5 harbors genes encoding a protein that is translocated by the SPI-1-encoded T3SS, as well as for a protein translocated by the SPI-2-encoded T3SS (Knodler et al., 2002). There are also a large number of substrate proteins of the T3SS of SPI-1 and SPI-2 that are encoded by several distinct loci outside of SPI. These loci do not meet the criteria of PAI; however, characteristic features of horizontally acquired DNA are present such as bacteriophage genes. The SPI-1- and SPI-2-encoded T3SS encode the basic machinery for protein translocation, and acquisition of additional virulence determinants by horizontal gene transfer allowed the formation of strains with different pathogenic potentials and their adaptation to various hosts. This situation is depicted in Figure 3. Further information on SPI can be found in recent reviews (Hansen-Wester and Hensel, 2001; Marcus et al., 2000).

In addition to SPI-1 and SPI-5, several other PAI-like elements have been identified by the genome-sequencing projects of *S. enterica* serovars Typhi and Typhimurium, as well as by specific screens (McClelland et al., 2001). Yet the contribution of these further elements to the pathogenesis of *Salmonella* infections needs to be analyzed.

6.5. *Pathogenicity Islands in* Staphylococcus aureus

S. aureus PAIs harbor a large number of allelic forms of exotoxins or secreted proteins. A set of antibiotic resistance genes is clustered within a resistance island in methicillin-resistant *S. aureus* (MRSA). Although most PAIs have been identified in Gram-negative pathogens, there are an increasing number of examples for PAIs in Gram-positive pathogens. An important Gram-positive pathogen is *S. aureus*, a species that can be found as a commensal on human body sites, as well as the causative agent of skin infections and severe diseases such as septicemia, endocarditis, toxic shock syndrome, and many more. *S. aureus* frequently causes nosocomial infections, and a major problem for the treatment of these infections is the resistance against a variety of antibiotics, e.g., the β-lactam antibiotic methicilllin. Analyses of the

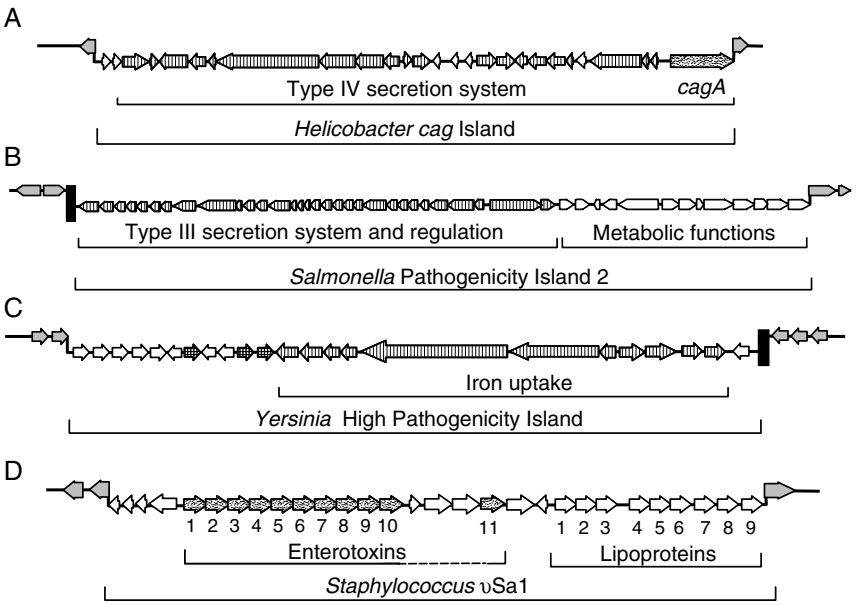

FIGURE 2. Several PAIs can interact in a complex network of virulence functions. This example shows the relationship of the functions of the PAIs SPI-1, SPI-2, and SPI-5, which each play important roles in the virulence of *Salmonella enterica*. The large PAI SPI-1 and SPI-2 each encode a type III secretion system (T3SS), complex assemblies in the cell envelope. In addition, substrate proteins that are secreted or translocated by the SPI-1-encoded T3SS or the SPI-2-encoded T3SS are encoded by the respective PAI. Further substrate proteins of either system are encoded by various gene loci outside of SPI-1 or SPI-2. SPI-5 harbors genes encoding substrate proteins for the SPI-1 system as well as for the SPI-2 system. Finally, there are additional loci outside of the PAI that encode for substrate proteins that can be secreted by the SPI-1 system as well as by the SPI-2 system. SPI-1 and the cognate substrate proteins function in the invasion of eukaryotic host cells and enteropathogenesis. SPI-2 and the substrate proteins of the T3SS are important for systemic pathogenesis of *S. enterica* and intracellular survival and replication.

resistance gene in MRSA indicated the location within a locus termed "*Staphylococcus* cassette chromosome *mec*" (SCC_{mec}). Several allelic forms of methicillin-resistance genes and genes encoding resistance against other antibiotics are present in SCC_{mec}. In addition to SCC_{mec}, other genomic islands such as νSaα and νSaβ that match the criteria of PAIs were identified by genome analyses (Baba et al., 2002). These PAIs contain genes encoding secreted virulence determinants such as exotoxins or proteases. Interestingly, several allelic forms of these virulence genes were found in repetitive arrangements in the PAI. The presence of the allelic forms of virulence genes may be a consequence of the pathogenic flexibility of *S. aureus* and enable the pathogen to colonize different body sites and tissues of an infected organism.

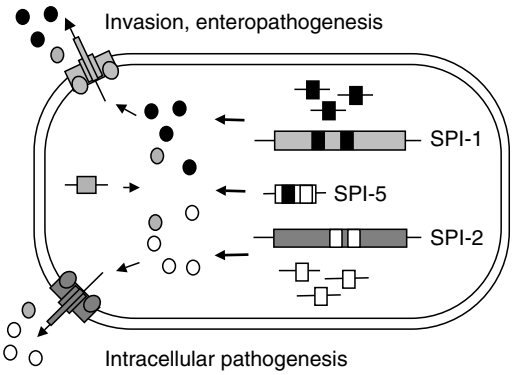

FIGURE 3. Topologies for PAIs of various pathogens are shown. (A) The *cag* island of *Helicobacter pylori* harbors genes for a type IV secretion system (T4SS) that can mediate the translocation of the effector protein CagA (red) into eukaryotic cells. (Modified from (Fischer et al., 2001.) (B) The High Pathogenicity Island (HPI) of *Yersinia enterocolitica* is an example of an instable PAI. Several insertion sequence elements are present within this PAI. Genes in HPI encode a high-affinity iron uptake system, which is important for the extracellular proliferation of the pathogen during colonization of the host. (Modified from Carniel, 2001.) (C) *Salmonella* Pathogenicity Island 2 (SPI-2) shows a mosaic structure. SPI-2 has been defined as a genetic element of about 40 kb that is absent from the related species *Escherichia coli*. Only a portion of 25 kb is required for systemic infection and encodes for a type III secretion system (T3SS), secreted proteins, and regulatory proteins. Another portion of 15 kb is not required for virulence, and harbors genes for metabolic or unknown functions (open symbols), such as an enzyme system for alternative electron acceptors during anaerobic growth. (Modified from Hansen-Wester and Hensel, 2001.) (D) The νSa1 PAI of methicillin-resistant *Staphylococcus aureus* (MRSA). (Modified from Baba et al., 2002.) A remarkable feature of PAIs in Gram-positive pathogens is the presence of a large number of genes with related functions, such as genes for enterotoxin or lipoproteins.

There are no indications of protein translocation systems encoded by PAIs of *S. aureus*, which is in line with the observation that most virulence factors are either soluble extracellular proteins or components that are bound to the bacterial cell surface (see Fig. 2d). (See Novick et al., 2001 for further details and reference to original publications.)

7. Specific Aspects of Pathogenicity Islands

7.1. Mosaic Pathogenicity Islands

PAIs can appear as single integrations and also as mosaic assemblies of several elements that have been successively acquired by horizontal gene transfer. Some PAIs represent an insertion of a single genetic element. Other

PAIs show a more complex structure, as elements of different origin are present (Figure 1E). During evolution, several genetic elements have been acquired independently at different time points and from different hosts. However, these DNA acquisitions are often integrated at the same position into the chromosome of the recipient bacteria. This will result in the accumulation of horizontally acquired elements at a certain location of the chromosome, and the same target structures (e.g., tRNA genes) served repeatedly for the integration of the various elements.

7.2. Regulation of Virulence Genes in Pathogenicity Islands

Virulence genes in PAIs are frequently under direct control of regulators also encoded by the PAI. In addition, the expression is modulated by global regulatory systems encoded by the core genome. With a few exceptions, the expression of virulence genes is highly regulated and responds to specific factors encountered by the pathogen within the host organism. Complex regulatory networks exist that encode regulators directly controlling the expression of a single, or a small group of, virulence gene(s) as well as global regulators that control the coordinate expression of a large number of virulence functions. This phenomenon is also observed for virulence genes in PAIs. Often, specific regulators are encoded by genes within the PAI that control the expression of other genes within the PAI. The genes for such local regulators have been acquired together with structural genes for virulence functions. However, in the course of integration into the host genome, regulatory control over the horizontally acquired virulence genes was obtained, and PAI-encoded functions were expressed in concert with other virulence factors.

7.3. Black Holes: Virulence Due to the Lack of Genes?

Not only the integration of new genes but also the loss of certain metabolic functions can increase bacterial virulence. Not all virulence characteristics of a pathogen can be explained by the acquisition of additional genes. There is also evidence that the absence of specific gene clusters increases the pathogenic potential of bacteria. The comparison of nonpathogenic *E. coli* strains to *Shigella* spp. and enteroinvasive *E. coli* revealed the absence of large chromosomal regions harboring the *cadA* for lysine decarboxylase (Maurelli et al., 1998). The experimental introduction of *cadA* in the pathogenic strains and species attenuated virulence, indicating that the gene function is not compatible with pathogenic lifestyle. This observation indicates that bacterial pathogenicity is also dependent on a finely tuned ratio between virulence factors and basic metabolic functions.

7.4. Do All Pathogens Possess Pathogenicity Islands?

PAIs are important elements of pathogenicity in a large number of bacterial pathogens, but there are also important pathogens without PAIs. Given the frequent presence of PAIs in a large number of pathogens, one may ask if

PAIs are a general theme in bacterial pathogenesis. In other words, can bacteria be pathogenic without PAIs? Genome analyses indicated that several important pathogens lack genomic elements that resemble PAIs. Genomic regions that show typical characteristics of PAIs were not detected in important pathogens such as *Mycobacterium tuberculosis, Borrelia burgdorferi,* or *Streptococcus pyogenes*.

Several reasons can be considered for the absence of PAIs in certain pathogens:

(1) Some bacterial pathogens show an extreme reduction of genome size, which is often a consequence of obligate parasitism. These bacteria are dependent on several functions provided by the host organisms. Often, these pathogens also lack complex virulence functions.
(2) Due to a unique lifestyle, such as obligate intracellular parasitism, some bacterial pathogens are excluded from the common gene pool and may not have access to virulence genes from other bacteria.
(3) Other bacterial pathogens show a very high rate of reorganization of the genome or even of individual genes. These pathogens may have acquired PAIs, but the rapid reorganization makes it difficult to detect horizontally acquired DNA elements.

7.5. Genomic Islands in Nonpathogenic Bacteria and "Fitness Islands"

Insertions of large blocks of genes or genomic islands are also observed in nonpathogenic bacteria and allow the acquisition of metabolic functions. The acquisition of large genomic fragments is a mechanism of evolution that is not delimited to pathogenic bacteria, but a rather common phenomenon. In a large number of nonpathogenic, commensal or environmental bacteria, chromosomal insertions can be observed that have characteristics similar to PAI; however, virulence genes are absent in these loci. These loci have been termed "genomic islands" and the acquisition and integration of these elements is likely to be similar to that of PAI. Genomic elements termed "fitness islands" have intermediate characteristics. These insertions that are found in pathogenic as well as in nonpathogenic bacteria do not contain specific virulence genes but rather encode factors that enhance the fitness of the organisms in colonization of environmental niches. Such loci may encode additional metabolic pathways or systems with redundant functions to those encoded by the core genome.

8. Conclusions

In a large number of bacterial pathogens PAIs carry important virulence functions. Horizontal transfer of PAIs allows rapid transfer of complex virulence traits between species and strains, leading to "quantum leaps" in evolution. A large number of bacterial virulence functions are encoded by genes

clustered within distinct regions of the chromosome that were termed Pathogenicity Islands (PAIs). Many PAIs have features of mobile genetic elements and thus it is not surprising that horizontal gene transfer led to the distribution of PAIs among a variety of bacteria. The distribution of PAIs among bacteria allows the rapid spread of virulence functions and the development of bacteria with new or extended pathogenic potential. The acquisition of virulence functions by horizontal gene transfer of PAIs has been coined "quantum leaps" in bacterial evolution. Often, complex virulence functions such as protein translocation systems are encoded by PAIs, but it is also observed that PAIs harbor a collection of allelic forms for a virulence factor such as an exotoxin. Genetic elements that promote the horizontal distribution of PAIs and their integration into the host genome also contribute to the genetic instability of PAIs and can promote their loss. On the other hand, there is an evolutionary pressure to maintain PAIs that are essential for the pathogenic lifestyle of the bacterium, and genes promoting motility and instability of the PAI are inactivated and finally deleted. The identification and functional analyses of PAIs in known and new emerging pathogens will significantly improve our understanding of bacterial virulence.

Questions to Consider

1. List the common characteristics of PAIs.

Some or all of the following features can be observed in PAIs:

(1) Large genomic insertions containing virulence genes
(2) Specific insertion points
(3) Specific structural features such as base composition
(4) Genetic instability
(5) Characteristic point of insertion

2. How can PAIs promote the rapid evolution of bacterial virulence?

Complex virulence functions are transferred in a single step to a new host. New functions, such as proteins translocation systems or iron uptake systems, are rapidly available after integration of the PAI and will allow the recipient to colonize new hosts or niches within a host organism. The evolution of such functions de novo would require considerably longer periods of time.

3. Describe the contribution of bacteriophages to the evolution of bacterial virulence.

Many bacteriophages allow the replacement of a part of their genome by bacterial genes. This allows for bacterial virulence genes to be transferred by bacteriophages. There are several PAI that contain bacteriophage genes. A large number of bacteriophages are capable of generalized transduction. During replication, a fragment of the bacterial chromosome is erroneously

packed into a phage head. Since the resulting particle is still capable of infection, a section of the chromosome can be transduced into another bacterial cell and may integrate into the genome.

4. Describe evolutionary events from acquisition of a PAI to the integration into the core genome of a pathogen.

The possible sequence of events from acquisition to stable integration of a PAI are:

(1) Acquisition of a DNA fragment containing virulence genes by horizontal gene transfer
(2) Integration of a horizontally acquired element into the genome of the new host
(3) Mutational inactivation and/or deletion of genes involved in mobility and thus instability
(4) Deletion of genes on the PAI not relevant for the virulence function
(5) Amelioration of codon usage and base composition of PAI genes to the core genome

The PAIs of various pathogens introduced in this chapter reflect different stages of this evolutionary process.

5. Which prerequisites have to be met for the transfer of complex virulence functions in the form of a PAI?

All genes that are required for the virulence function have to be clustered within a distinct genetic element. If the virulence trait requires the function of three genes, it is more likely that the virulence trait is transferred if the genes are arranged in an operon, rather than distributed over the chromosome. In fact, many genes in PAIs are organized in operons. Furthermore, the new host of the PAI needs to be able to express the PAI-encoded function. Thus, the codon usage and structure of regulatory elements of genes in PAIs must not be too different from that of the host genome.

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Chapter 5

Capsules

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Historical Landmarks

1881 Pasteur describes light microscopy observations of substance ("aureole") surrounding *Streptococcus pneumoniae* (Pasteur, 1881).

Late1800s to early 1900s Recognition of the requirement for capsule in the pathogenesis and resistance to phagocytosis of many bacteria.

1925 Recognition of the polysaccharide nature of capsules (Avery and Morgan, 1925; Heidelberger et al., 1925).

1928 The encapsulated phenotype of *S. pneumoniae* is used by Griffith to demonstrate transformation in the first observation of genetic exchange between bacteria (Griffith, 1928).

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1944 Avery et al. (1944) show that Griffith's "transforming principle" is DNA and lay the foundation for the field of molecular genetics.

1945 Protective effects of immunization with serotype-specific capsules are demonstrated in humans (MacLeod et al., 1945).

1950s and 1960s Genetic analyses of capsule genes and biochemical analyses of enzymes involved in capsule synthesis (Mäkelä and Stocker, 1969; Markovitz, 1977).

1980s to the present Molecular characterization of bacterial capsule loci (Roberts, 1996; Whitfield and Roberts, 1999; Yother, 1999).

1987 Introduction of *Haemophilus influenzae* type b conjugate vaccine (Adams et al., 1993).

1990s to the present Determination of the complete nucleotide sequences of numerous bacterial capsule loci (Amor and Whitfield, 1997; Chaffin et al., 2000; DeAngelis et al., 1993; Dillard et al., 1995; Drummelsmith and Whitfield, 1999; Kolkman et al., 1997b; Morona et al., 1997; Pazzani et al., 1993; Petit et al., 1995; Satola et al., 2003; Sau et al., 1997; Stevenson et al., 1996).

1990s to the present Molecular and biochemical characterization of the mechanisms of capsule synthesis (Roberts, 1996; Whitfield and Roberts, 1999).

1. Introduction

During the course of infection, bacteria are constantly bombarded by the host's immune system. Antibodies, complement, and a variety of immune cells strive to rid the host of the invading bacteria. One means employed by many bacteria to survive within this hostile environment is the production of a capsule or exopolysaccharide (EPS). This diverse group of macromolecules makes up the outer surface of the bacterium and is the first line of defense against the host's immune system. Capsules are usually composed of polysaccharides that are covalently attached to the cell surface, whereas EPS are released into the surrounding environment. Despite the difference in the final locations of capsules and EPS, they have similar mechanisms of synthesis and can be structurally related. Most capsules and EPS are composed of repeating oligo- or monosaccharides. In a few bacteria, however, the capsule is polypeptide in nature.

Besides playing a role in the pathogenesis of many bacteria, capsules have also been central to several important scientific discoveries. In 1928, Griffith took advantage of the essential nature of the *Streptococcus pneumoniae* capsule to demonstrate the transfer of capsule phenotypes between bacteria (Griffith, 1928). In these experiments, Griffith showed that a nonencapsulated, avirulent strain of pneumococcus was converted to an encapsulated,

virulent phenotype when coinfected in mice with a heat-killed encapsulated strain. In 1944, Avery et al. expanded on Griffith's work and demonstrated that the material exchanged between the bacteria was DNA (Avery et al., 1944) (see Chapter 2). The genes required to synthesize *S. pneumoniae* capsules are found in a single locus on the bacterial chromosome, a fact that contributed to the success of these experiments. Molecular and genetic characterization of capsules in other bacterial pathogens has also shown that the capsule genes usually reside in a single locus. Differences in the organization and genetic content of these loci allow for a high degree of heterogeneity in capsule structures and compositions. Because of the vast number of monosaccharides available for use in capsule synthesis and the varying glycosidic linkages that can be formed between two sugars, capsules are the most diverse structures on the bacterial surface. This diversity is most evident in *Escherichia coli* and *S. pneumoniae*, where 80 and 90 serologically distinct capsule types, respectively, have been identified. Since capsules are often the serologically distinguishing feature of the bacterium, typing systems based on distinct seroreactivity are used for classifying many encapsulated bacteria.

Although not all pathogenic bacteria produce a capsule, for the ones that do, the capsule usually contributes to the virulence of the bacterium. Listed in Table 1 are some common bacterial pathogens, the number of serologically distinct capsules they produce, and the role the capsule plays in the pathogenesis of the bacterium. The purpose of this chapter is to give an overview of the current knowledge on capsules. Because the biochemistry and genetics of capsule synthesis in *E. coli* and *S. pneumoniae* have been well characterized, they serve as good models for understanding capsules in Gram-negative and Gram-positive bacteria, respectively. We therefore focus much of our attention on these organisms.

2. Roles of Capsules in Pathogenesis

Capsules can play a variety of roles in bacterial pathogenesis, including the inhibition of phagocytosis, the prevention of complement-mediated lysis, adherence to host cells, and contribution to the formation of abscesses and other inflammatory processes. These roles can be fulfilled in different ways by different bacteria expressing structurally similar capsules, indicating that capsule alone may not be the sole determinant in these processes. In some bacteria, such as *S. pneumoniae*, the production of a capsule is an absolute requirement for virulence, as described below. In others, such as *S. pyogenes* (Group A *Streptococcus*) and *Staphylococcus aureus*, it is an accessory factor that enhances, but is not essential for, virulence (Moses et al., 1997; O'Riordan and Lee, 2004). Capsules may also be important in allowing the bacterium to colonize its host without causing overt disease.

TABLE 1. Capsules of pathogenic bacteria.

Bacterium	Number of capsular serotypes	Frequent types in infections[a]	Function[b]	Biosynthetic mechanisms[c]
Bacillus anthracis	1 (polyglutamic acid)		Antiphagocytic	
Escherichia coli	80	K1, K5	Antiphagocytic	1, 2
Haemophilus influenzae	6	b	Antiphagocytic	2
Klebsiella pneumoniae	77	K1, K2	Antiphagocytic/ antilytic	1
Neisseria meningitidis	12	A, B, C	Antiphagocytic/ antilytic	2
Pseudomonas aeruginosa	1 (alginate)		Antiphagocytic	
Salmonella enterica typhi	1 (Vi antigen)		Antiphagocytic/ antilytic	
Staphylococcus aureus	11	5, 8	Antiphagocytic	1
Streptococcus agalactiae	9	Ia, III, V	Antiphagocytic	1
S. pneumoniae	90	Children: 4, 6B, 9V, 14, 18C, 19F, 23F Adults: 1, 3, 4, 6, 7, 8, 9, 12, 14, 18, 23	Antiphagocytic	1, 3
S. pyogenes	1 (hyaluronan)		Antiphagocytic/ adherence	3

[a] With reference to invasive disease; indicated for bacteria that have more than one serotype.
[b] Antiphagocytic capsules inhibit deposition of complement or block access of phagocytic receptors to deposited complement. Antilytic capsules prevent bacterial lysis by the complement C5–9 membrane attack complex. Additional functions have been proposed for many of the capsules.
[c] Mechanisms are those that have been described or can be predicted from protein sequence. Additional mechanisms may occur in serotypes that have not been characterized. 1, block-type (Wzy-dependent); 2, ABC-2 transporter-dependent; 3, synthase-dependent.

The requirement for capsules in *S. pneumoniae* virulence was strongly suggested by many early experiments demonstrating that spontaneously derived, nonencapsulated mutants were avirulent in animal models (Eyre and Washbourn, 1897; Griffith, 1928; Stryker, 1916). Enzymatic removal of the *S. pneumoniae* type 3 capsular polysaccharide confirmed the essential nature of the capsule in virulence (Avery and Dubos, 1931), and genetic analyses made possible by the molecular characterization of capsule loci provided unequivocal demonstrations of the requirement for capsules for the virulence of many other bacteria (Hardy et al., 2001; Moxon et al., 1984; Wessels et al., 1989, 1994). Despite the great diversity in capsule structures, strains expressing particular capsular serotypes often predominate in specific diseases. For example, 90 serotypes have been identified among *S. pneumoniae* isolates, but the majority of infections are caused by strains expressing 1 of only 23 serotypes (Butler et al., 1995; Finland and Barnes, 1977; Gray and H.C. Dillon, 1986;

Lindberg, 1999a). Further, a particular subset of these types occurs primarily in pediatric infections, whereas a different subset occurs in adult infections (Table 1). For *S. aureus*, which produces 11 capsule types, only 1 or 2 of these are frequently found in clinical isolates (O'Riordan and Lee, 2004). *Haemophilus influenzae*, a major cause of meningitis in children, produces 6 capsule types, yet only capsule type b is found on strains that cause invasive disease (Lindberg, 1999a). Similarly, *E. coli* strains causing neonatal meningitis predominantly express the K1 capsule (Siitonen et al., 1993).

Studies in *S. pneumoniae* and *Klebsiella pneumoniae* have used genetic exchange of capsule loci to switch the type of capsule expressed (Kelly et al., 1994; Ofek et al., 1993). The results showed that the capsule type can have a major effect on the virulence of the organism but that the effect is dependent on the combination of capsule type and other factors in the genetic background. So why do bacteria maintain such a high level of capsule diversity? For many bacteria, the answer to this question may not reside totally in the role of capsule in disease but also in additional ecological niches the bacteria may inhabit.

2.1. Capsules May Interfere with Complement-mediated Effects

The complement system plays an important role in opsonophagocytosis of both Gram-positive and Gram-negative bacteria and in lysis of Gram-negative bacteria. Consequently, encapsulated bacteria are frequent causes of infections in patients with diseases resulting in complement deficiencies. Capsules are generally poor activators of the complement cascade; however, they may inhibit complement-mediated phagocytosis either by preventing deposition of complement fragments on the bacterial surface or by preventing phagocytic receptors from gaining access to bound complement. In Gram-positive bacteria, the alternative complement pathway is activated by cell wall components, resulting in the deposition of C3b and iC3b fragments on the cell wall. These fragments serve as opsonins that can be recognized by receptors on phagocytic cells, leading to phagocytosis of the bacteria. In *S. pneumoniae*, similar amounts of C3b are deposited on encapsulated strains and their isogenic non-encapsulated derivatives. However, the non-encapsulated strains are readily phaogocytized, whereas the encapsulated isolates resist phagocytosis. Here, the capsule serves largely to prevent access to bound complement, while to a lesser extent reducing the amount of complement that is deposited. All *S. pneumoniae* capsules function in a similar manner, but some serotypes are more effective than others in this regard (Abeyta et al., 2003; Hostetter, 1986; Winkelstein et al., 1980). Reducing access to bound complement is also an important function of *S. aureus* capsules, but here the capsule plays a greater role in preventing complement deposition (Cunnion et al., 2003). In *S. agalactiae* (Group B *Streptococcus*) type III, the sialic acid residues of the capsule function to prevent the activation and deposition of complement on the cell wall (Marques et al., 1992).

In Gram-negative bacteria, complement activation by surface structures, such as lipopolysaccharide (LPS), can lead to opsonophagocytosis, as described above. Alternatively, the terminal components of the complement pathway (C5–9) may assemble to form the membrane attack complex (MAC). Insertion of the MAC through the membrane leads to bacterial lysis. In Gram-positive bacteria, the lytic attack is generally prevented due to the thick cell wall, which prevents insertion of the MAC into the cytoplasmic membrane. In the Gram-negative group B *Neisseria meningitidis*, the polysialic acid capsule blocks insertion of the MAC (Ram et al., 1999), whereas in *K. pneumoniae*, the capsule reduces complement deposition and thereby reduces both complement-mediated killing and opsonophagocytosis (Alvarez et al., 2000).

In addition to impeding access to surface structures that activate the alternative complement pathway, capsules can also reduce binding of specific antibodies to surface antigens, thereby reducing activation of the classical complement pathway.

2.2. Capsules Are Mediators of Adherence and Colonization

Capsules may play important roles in mediating attachment to host cells, or they may interfere with adherence as a result of blocking access of other surface-localized bacterial adhesins to eukaryotic receptors. In vitro, nonencapsulated derivatives often adhere more firmly to eukaryotic cells than their encapsulated parents (e.g., with *N. meningitidis*, *S. pneumoniae*, *S. aureus*, and *H. influenzae*) (Pohlmann-Dietze et al., 2000; Read et al., 1996; St Geme and Falkow, 1991; Talbot et al., 1996). Adherence is an important part of colonization and a necessary first step in many infections; thus, for bacteria in which the capsule itself does not act as an adhesin, expression of the capsule is expected to be reduced in environments where adherence is essential to bacterial survival (see Chapter 7). In respiratory tract colonization by *S. pyogenes*, the hyaluronan (HA) capsule mediates attachment to epithelial cells via interaction with CD44, a hyaluronic acid binding protein, and also protects against immune clearance (Ashbaugh et al., 2000). Sustained colonization of the nasopharynyx by *S. pneumoniae* also requires capsule production, although reduced levels appear to be adequate (Magee and Yother, 2001). Whether the *S. pneumoniae* capsule is involved in attachment to host cells or is needed to protect against the host's immune system is not known. The glucan polymers of *S. mutans* are examples of EPS that mediate adherence, in this case to the tooth surface, which may ultimately result in dental caries (Kuramitsu, 1993). Capsule-deficient mutants of *E. coli*, *N. meningitidis*, and *K. pneumoniae* exhibit reduced colonization in animal models, indicating a role for capsules in these processes (Favre-Bonte et al., 1999; Herias et al., 1997; Yi et al., 2003). In addition, antibodies to capsules can reduce the number of bacteria colonizing the nasopharyngeal and oropharyngeal surfaces, as observed with *S. pneumoniae* and *H. influenzae* type b, respectively, further

demonstrating that capsules are expressed in these environments (Dagan et al., 1997; Malley et al., 1998; Takala et al., 1991).

2.3. Capsules Participate in a Variety of Other Functions

In many bacteria, capsules are important in the formation of biofilms, which are colonies of bacteria interconnected into a film-like layer by extracellular polysaccharides and proteins. Biofilms can be quite complex, containing channels for the funneling of waste and nutrients. Bacteria present in biofilms tend to be more resistant than free-living or planktonic cells to antibiotics and host defenses, making them more difficult to eradicate. *S. aureus* and *Pseudomonas aeruginosa* are two medically important examples of bacteria whose capsules contribute to biofilm formation (Dunne, 2002). Zwitterionic capsules, which carry both a positive and a negative charge, contribute to abscess formation by *Bacteroides fragilis* and *S. aureus* (Coyne et al., 2001; Tzianabos et al., 2001). Lastly, since most capsules are hydrophilic, they can absorb large quantities of water and can serve to prevent desiccation of the bacterium.

2.4. Capsules Can Elicit Protective Immune Responses

The host immune response may produce antibodies that bind specifically to the capsule. These antibodies are usually protective because they direct deposition of C3 fragments on the capsule itself, a location that is accessible to phagocytic receptors. For this reason, many bacterial vaccines are composed of capsular polysaccharides. One of the current vaccines for the Gram-positive human pathogen *S. pneumoniae* comprises the capsular polysaccharides of the 23 most common serotypes seen in infection. Like most purified polysaccharides, the *S. pneumoniae* capsules elicit only T cell-independent immune responses. Although protective, these immune responses result in lower levels of antibody than T cell-mediated responses, and they lack a strong memory response (Lesinski and Westerink, 2001). Additionally, certain populations, including infants younger than about 2 years of age, fail to mount adequate T cell-independent responses and therefore respond poorly to these vaccines. For polysaccharide-based vaccines targeting these populations, the polysaccharides are conjugated to a protein carrier, which results in a T cell-dependent response and therefore increased efficacy in children. Conjugate vaccines for *H. influenzae* type b comprise the polyribosylribitol phosphate type b capsular polysaccharide conjugated to one of several carriers, such as a diphtheria toxoid-like protein (cross-reactive material 197 [CRM197]) or a meningococcal outer membrane protein. These vaccines have been in widespread use in North America since 1991 and have resulted in a >97% reduction in meningitis due to *H. influenzae*, thus eliminating this pathogen as the major cause of childhood bacterial meningitis (Lindberg, 1999a). More recently, the introduction of an *S. pneumoniae* conjugate vaccine, comprising the

polysaccharides from the seven capsule types occurring most frequently in childhood invasive disease and conjugated to CRM197, has led to significant reductions in pneumococcal disease (Darkes and Plosker, 2002).

Although capsules can successfully be used in vaccines, many capsules mimic or contain components found in host cell glycans. These structures, such as the sialic acid residues of the *E. coli* K1 and *N. meningitidis* serogroup B capsules, the heparin-like K5 capsule of *E. coli*, and the HA capsule of *S. pyogenes*, are poorly immunogenic and are potentially capable of eliciting cross-reactive antibodies that could damage the host (Lindberg, 1999b).

3. Genetics and Classification of Capsules

Capsules are most often composed of complex repeating oligosaccharides that contain several sugars linked together by a variety of glycosidic linkages. Given the structural complexity of capsules, their syntheses generally require the activity of a large number of enzymes, including glycosyltransferases, polymerases, and transport machinery. The genes that encode the enzymes necessary to produce capsules in bacteria are usually grouped into large biosynthetic operons that can contain 20 or more genes. Generally, only a single set of capsule genes is present in a given strain, leading to the expression of a single capsular serotype. For each bacterial species, the capsule genes reside at a specific site in the chromosome, although in a few bacteria, such as the lactococci, the genes are plasmid-encoded (van Kranenburg et al., 1997). For some bacteria, like *E. coli*, the organization and composition of the operons can vary from capsule type to capsule type, whereas in most Gram-positive bacteria, the organization is more conserved between capsule types and between bacterial species. In some cases, sugars incorporated into capsules are also found in other cellular structures, such as peptidoglycan and the teichoic and lipoteichoic acids of Gram-positive bacteria. The enzymes necessary for synthesizing these sugars may be encoded by genes that reside outside the capsule locus, and the sugars may be derived from cellular pools that serve multiple pathways (Crater et al., 1995; Hardy et al., 2000; Mollerach et al., 1998).

Over the last decade, the nucleotide sequences of numerous capsule loci (including those described in Amor and Whitfield, 1997; Chaffin et al., 2000; DeAngelis et al., 1993; Dillard et al., 1995; Drummelsmith and Whitfield, 1999; Kolkman et al., 1997b; Morona et al., 1997; Pazzani et al., 1993; Petit et al., 1995; Satola et al., 2003; Sau et al., 1997; Stevenson et al., 1996) have been determined. Consequently, it has become possible to divide capsules into groups based on both the genetic organization of their capsule loci and their mechanisms of synthesis (Table 1). Discussed below are the classification and genetics of capsule synthesis in *E. coli* and *S. pneumoniae*, which are representative of most bacteria in this regard.

3.1. *E. coli Produces a Number of Different Cell Surface Polysaccharides, Including Lipopolysaccharide, Enterobacterial Common Antigen, Colanic Acid (slime), and Capsule*

LPS is composed of three portions: (1) lipid A, which comprises the outer leaflet of the outer membrane and serves as the lipid anchor for LPS; (2) the core polysaccharide, which contains approximately 10 sugars that are generally species-specific; and (3) O antigen, which is composed of a repeating oligosaccharide or monosaccharide and is antigenically variable. Enterobacterial common antigen (ECA), as its name implies, is a common polysaccharide that is found not only in *E. coli* but also in other enterobacteriaceae. In *E. coli*, ECA is composed of the trisaccharide repeat [–3)-α-D-Fuc4NAc-(1–4)-β-D-ManNAc-(1–4)-α-D-GlcNAc-(1-]. ECA is not required for virulence of *E. coli* and its function is unknown. Colanic acid, which is composed of a complex repeating branched oligosaccharide, is an EPS that is produced only at temperatures below 30°C. It does not occur in all *E. coli* strains and is not a virulence factor. Colanic acid may, however, be important for survival outside the host. Although ECA and colanic acid do not vary between strains of *E. coli*, both LPS and capsules demonstrate high levels of diversity, with 153 different O antigens and 80 different capsule types or K antigens. *E. coli* isolates are serologically distinguished based on their different O (LPS) and K (capsular) antigens. Although this chapter is focused on capsules, the syntheses of O antigens, ECA, colanic acid, and capsules in *E. coli* are interrelated and use similar mechanisms. Relevant examples of mechanistic similarities of these cell surface polysaccharides are highlighted throughout this chapter.

The genes required to synthesize *E. coli* capsules are found in a single locus on the chromosome. Between capsule types, however, the specific genes and organization within these loci can vary. Based on both genetic and biochemical criteria, the capsules of *E. coli* have been divided into four groups, each of which will be discussed briefly below. For a more detailed review of the genetics and biochemistry of these four capsule groups, there are several excellent review articles (Roberts, 1996; Whitfield and Roberts, 1999).

3.2. *Group 1 Capsules Are Usually Composed of Relatively Complex Repeating Units that Have Multiple Sugars and Often Contain Side Chains*

Group 1 capsules can exist in two different forms that contain the same repeat unit. K_{LPS} contains short capsule chains (1–8 repeat units) attached to lipid A, whereas capsular K antigen (K_{CPS}) comprises high–molecular weight capsules attached to the bacterial surface by an unknown lipid anchor.

Group 1 capsules are epitomized by the K30 capsule of *E. coli* [–2)-α-D-Man-(1–3)-β-D-Gal-(1-) backbone with a β-D-GlcUA-(1–3)-α-D-Gal side chain linked at position 3 of the Mannose to position 1 of the Galactose] and also occur in *K. pneumoniae* and *Erwinia amylovora*, although the latter two bacteria do not make K_{LPS} (Whitfield and Paiment, 2003; Whitfield and Roberts, 1999). The genes required for group 1 capsule synthesis are found in a single genetic locus positioned upstream of the LPS O antigen biosynthetic locus *rfb* and proximal to the *his* operon at 45 min on the *E. coli* chromosome (Figure 1). The group 1 capsule loci are composed of two regions: one that is specific for the capsule type being synthesized and one that contains genes that are common to regulation and chain length determination in all serotypes (Figure 1). Both regions are transcribed from a single promoter located upstream of the common genes. The colanic acid biosynthetic genes reside in a similar location on the chromosome as the group 1 capsule genes (Figure 1) but are not considered a group 1 capsule because colanic acid is not attached to the cell surface and is not expressed at temperatures above

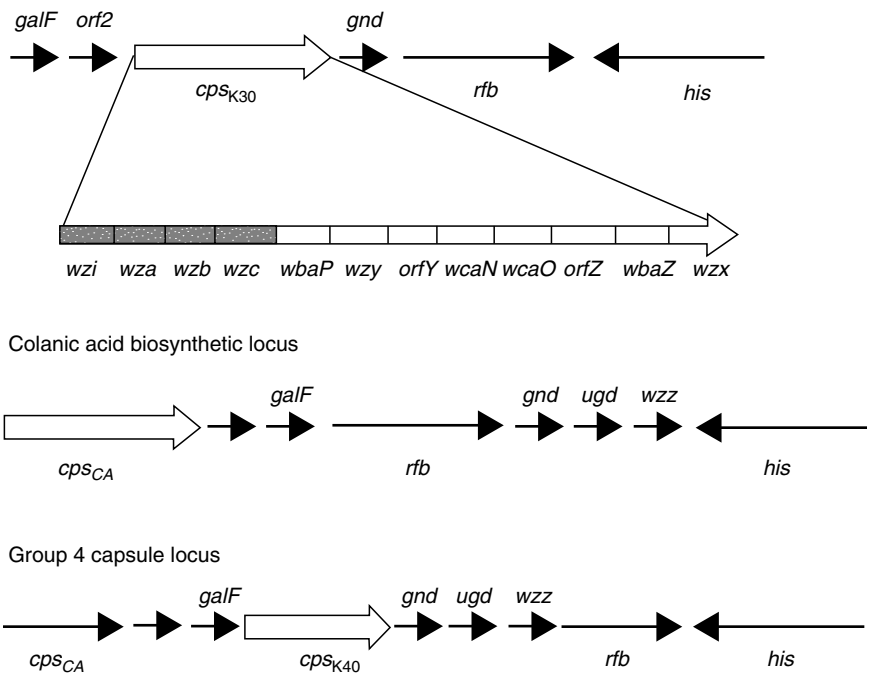

FIGURE 1. Genetic organization of the group 1 capsule, colanic acid, and group 4 capsule loci from Gram-negative bacteria. For the group 1 locus, shaded boxes indicate genes common among serotypes; open boxes indicate serotype-specific genes. Arrows indicate directions of transcription and putative transcripts. (Maps derived from Amor and Whitfield, 1997; Drummelsmith and Whitfield, 1999; Stevenson et al., 1996; Whitfield and Roberts, 1999.)

30°C (Markovitz, 1977). Like the group 1 capsule loci, the colanic acid biosynthetic locus also contains common genes upstream of the biosynthetic genes that are homologous to those in the group 1 capsule loci (Stevenson et al., 1996). *E. coli* strains that produce a group 1 capsule do not make colanic acid and express only a limited number of O antigen serotypes. Group 1 capsules are synthesized in a block-like manner where the repeating structure of the capsule is synthesized on a lipid carrier and subsequently polymerized. This mechanism of synthesis has been termed Wzy-dependent, since it involves a polymerase that is similar to the Wzy polymerase involved in O antigen synthesis, and is described in more detail below.

3.3. Group 2 and 3 Capsules Are Synthesized by a Similar Mechanism Utilizing an ATP-binding Cassette Type 2 (ABC-2) Transporter

Group 2 and 3 capsules utilize a very different mechanism of synthesis than that of group 1 capsules. Instead of a block-type mechanism, group 2 and 3 capsules are synthesized by a processive mechanism utilizing either a single glycosyltransferase or the concerted efforts of several glycosyltransferases. This mechanism also involves the use of an ABC transporter, as described in Section 4.2. Group 2 capsules are composed of relatively simple polysaccharides when compared to group 1 capsules. Examples of group 2 capsules are: K1, which is composed of polysialic acid; K4, which is composed of a chondroitin backbone [–4)-β-D-GlcUA-β(1–3)-β-D-GalNAc-β(1-], to which β-fructose is linked to position C-3 of the GlcUA residue; and K5, which is composed of [–4)-α-D-GlcUA-α-(1–4)-α-D-GlcNAc-β(1-] and is structurally similar to heparin.

The genes required for the synthesis of group 2 and 3 capsules are found in a single locus on the *E. coli* chromosome, mapping at 64 min and proximal to *serA*. The locus is divided into three separate regions, each with its own promoter and separate mechanism of regulation (Figure 2). The serotype-specific region 2, which contains the genes involved in synthesizing each individual polysaccharide, is flanked by regions 1 and 3, which are common to all capsule types and encode genes involved in transport and regulation. The main difference between group 2 and group 3 capsules is the organization of the common regions in the capsule loci, as shown in Figure 2. Additionally, group 3 capsule loci lack the gene encoding KpsU, the cytosine monophosphate 3-deoxy-manno-2-octulosonic acid (CMP-KDO) synthetase. CMP-KDO is critical for both group 2 and 3 capsule synthesis (as discussed below); however, the CMP-KDO synthetase for group 3 capsules is physically unlinked to the other genes associated with group 3 capsules. The CMP-KDO for group 3 capsules is believed to be provided by KdsB, an enzyme that is involved in the synthesis of the core polysaccharide of LPS. In addition, KdsB is also thought to provide CMP-KDO for group 2 capsule

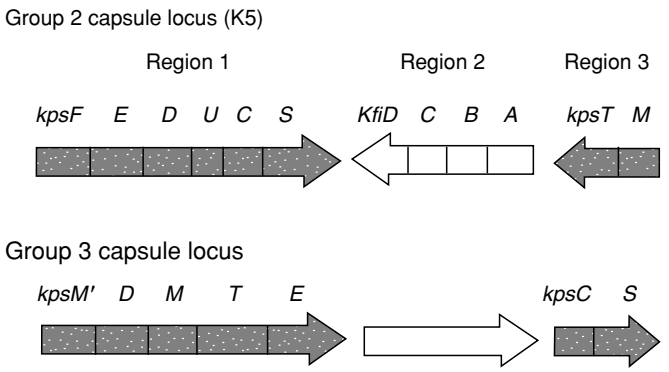

FIGURE 2. Genetic organization of the group 2 and group 3 capsule loci of Gram-negative bacteria. Shaded boxes indicate genes common among serotypes within a group; open boxes indicate serotype-specific genes. Arrows indicate directions of transcription and putative transcripts. (Maps derived from Pazzani et al., 1993; Petit et al., 1995; Whitfield and Roberts, 1999.)

synthesis, as mutation of *kpsU* has no effect on group 2 capsule synthesis (Bronner et al., 1993). The presence of KpsU, however, results in elevated CMP-KDO synthetase activity in group 2 capsule strains and is one of the criteria for separating capsule types into group 2 or group 3.

3.4. Group 4 Capsules Are Also Synthesized by a Wzy-dependent Mechanism

Group 4 capsules are referred to as O antigen capsules or amino capsules based on the fact that the repeating structure of the capsule often mimics the structure of a known LPS O antigen and because they contain amino sugars such as GlcNAc or GalNAc. For example, the K40 capsule of *E. coli* is composed of the linear backbone [–6)-α-D-GlcNAc-(1–4)-β-D-GlcA-(1–4)-α-D-GlcNAc-(1-] with an L-serine linked at position 6 of the GlcA residue. Group 4 capsules, like group 1 capsules, are synthesized by a block-type mechanism and are expressed with a limited range of O antigen serotypes. Much like group 1 capsules, group 4 capsules can also be found in a K_{LPS} or K_{CPS} form. Because of the difficulty in distinguishing serologically between O antigen capsules and LPS O antigens, the prevalence of O antigen capsules likely has been overlooked. Like the group 1 capsule loci, the genes required to synthesize group 4 capsules are found at 45 min on the *E. coli* chromosome and are found upstream of the *rfb* locus. In many group 4 strains, however, there is also a colanic acid biosynthetic locus upstream of the group 4 capsule locus (Figure 1). Thus, unlike *E. coli* strains that produce group 1 capsules, those producing group 4 capsules are able to synthesize colanic acid in addition to a capsule and an O antigen. Group 4 capsule loci do not contain a common region, but

synthesis of all group 4 capsules requires the activity of enzymes encoded by genes outside the capsule locus, including the O antigen-related enzymes WecA, WaaL, and Wzz, which are described below (Amor and Whitfield, 1997).

3.5. The Capsules of Gram-positive Bacteria such as Streptococcus pneumoniae Demonstrate as Much Diversity as Escherichia coli, but They Exhibit Less Genetic Heterogeneity

Most of the genes involved in Gram-positive capsule synthesis reside within a single locus under the control of a single promoter (Figure 3; reviewed in Yother, 1999). There are, however, two different mechanisms of synthesis for Gram-positive capsules, and the characterized capsule types have been grouped according to this difference. Most Gram-positive capsules are synthesized by the Wzy-dependent block-type mechanism, whereas a few utilize a processive mechanism, as described in Section 4. The capsule loci comprise: (1) a type-specific region, which contains the genes that encode the glycosyltransferases, polymerases, and other enzymes necessary for synthesis of each specific capsule structure; and (2) the common regions, which flank the type-specific genes and are found in all capsular serotypes. Contained within the upstream common region of most loci are genes involved in the regulation or modulation of capsule synthesis. These genes are fairly conserved among Gram-positive bacteria, although the order of the genes can vary. In *S. pneumoniae*, the cassette-like organization of the capsule loci allows the common genes and other homologous sequences flanking the serotype-specific genes to serve as sites for genetic exchange between the resident capsule locus and that contained in donor DNA obtained during transformation (Dillard and Yother, 1994). Genetic switching of capsule types was the basis for Griffith's original observation of gene exchange (Griffith, 1928) and is evident among clinical isolates of *S. pneumoniae* and *N. meningitidis* (Coffey et al., 1998; Swartley et al., 1997). Not only might this exchange provide a means for *S. pneumoniae* to acquire a more protective capsule type but it might also be one mechanism contributing to high levels of capsule diversity.

4. Mechanisms of Capsule Synthesis

The syntheses of all capsular polysaccharides begin with the activation of sugars, usually by the addition of a nucleotide to form a nucleotide diphosphate sugar. These activated sugars are then used by glycosyltransferases to form either individual repeating subunits or the long chain capsular polysaccharide. Most of the information on the biochemistry of capsule synthesis in bacteria comes from studying capsule and LPS O antigen synthesis in Gram-negative bacteria. For O antigens, three mechanism of synthesis have been

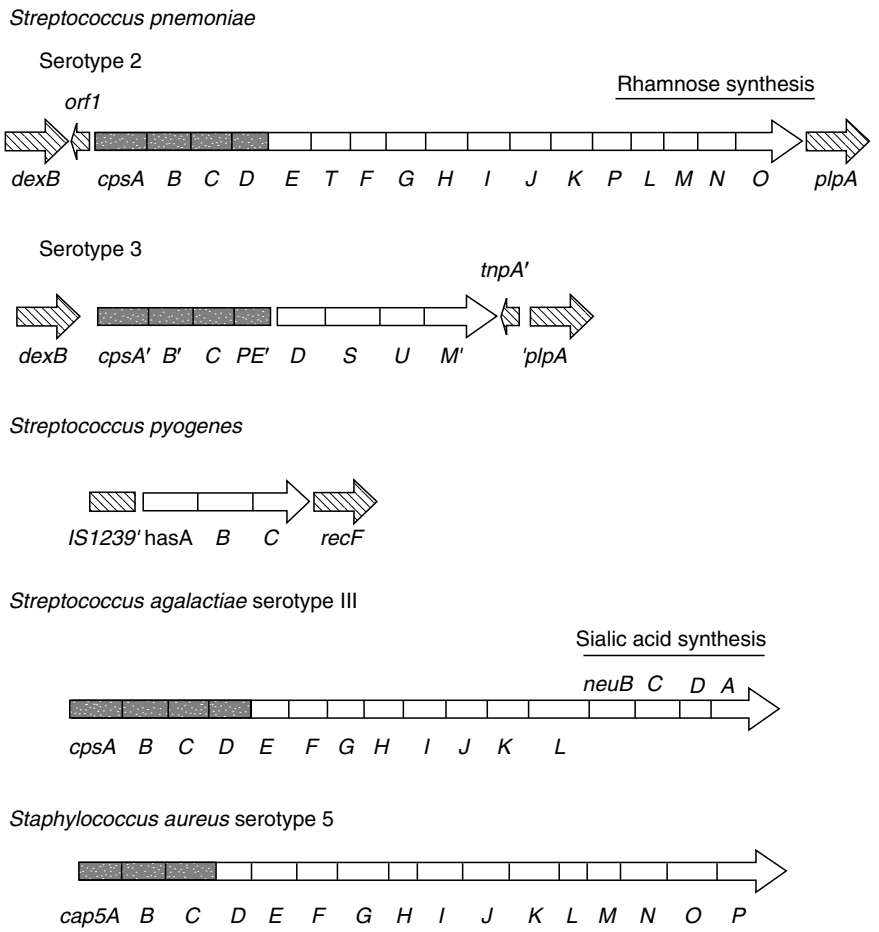
Streptococcus pnemoniae
Serotype 2
orf1
Rhamnose synthesis
dexB cpsA B C D E T F G H I J K P L M N O plpA
Serotype 3
tnpA'
dexB cpsA' B' C PE' D S U M' 'plpA
Streptococcus pyogenes
IS1239' hasA B C recF
Streptococcus agalactiae serotype III
Sialic acid synthesis
neuB C D A
cpsA B C D E F G H I J K L
Staphylococcus aureus serotype 5
cap5A B C D E F G H I J K L M N O P

FIGURE 3. Genetic organization of capsules from Gram-positive bacteria. Shaded boxes indicate genes that are common among all the loci (although the *S. aureus* common genes are in a different order); open boxes indicate serotype-specific genes; hatched boxes represent genes flanking the capsule loci. Arrows indicate directions of transcription and putative transcripts. The serotype 2 locus is representative of the majority of *S. pneumoniae* capsule types. The common region of the *S. pneumoniae* serotype 3 locus is neither transcribed nor required for capsule production. The *S. pneumoniae* serotype 3 *cpsD*, *cpsS*, and *cpsU* are homologous to the *S. pyogenes hasB*, *hasA*, and *hasC*, respectively. Synthesis of the *S. pneumoniae* serotype 3 and the *S. pyogenes* HA capsules utilizes a synthase-dependent mechanism, whereas the other capsules utilize a block-type mechanism, as described in the text. (Maps derived from Arrecubieta et al., 1995; Caimano et al., 1998; Chaffin et al., 2000; Crater et al., 1995; DeAngelis et al., 1993; Dillard et al., 1995; Iannelli et al., 1999; Sau et al., 1997.)

described (Raetz and Whitfield, 2002): (1) a block-type mechanism involving a Wzy-dependent pathway; (2) an ABC-2 transporter-dependent pathway; and (3) a synthase-dependent pathway. Capsule synthesis can also be grouped

into these same categories. All three mechanisms have been observed in Gram-negative bacteria, whereas only the Wzy-dependent and synthase-dependent pathways have been observed in Gram-positive bacteria.

4.1. The Block-type or Wzy-dependent Mechanism of Synthesis

The block-type mechanism is the most common pathway used in the synthesis of capsules in bacteria. This mechanism is most often used in capsules that have complex repeat units comprising multiple sugars and, often, side chains. It requires multiple glycosyltransferases to form all the glycosidic linkages between the individual sugars, as well as a separate polymerase (Wzy in Gram-negative bacteria) to polymerize the repeat units into polysaccharide. In *E. coli*, the block-type mechanism is used in the synthesis of group 1 and group 4 capsules. A list of the common enzymes used in the Wzy-dependent pathway, along with their functions and counterparts in *S. pneumoniae* and *S. aureus* capsule synthesis, is found in Table 2. For the most part, this pathway is the same for both Gram-negative and Gram-positive bacteria. Three conserved steps are involved: (1) initiation of synthesis and formation of a repeat unit; (2) transport of the repeat unit to the outer surface of the cytoplasmic membrane; and (3) polymerization of the repeat units into polymers (Figure 4). Following polymerization, the pathway differs between Gram-negative and Gram-positive bacteria. Gram-negative bacteria transport the completed polysaccharide chains across the outer membrane and anchor them to the outer face of the membrane via attachment to lipid A (K_{LPS}) or a glycerophosphate lipid (K_{CPS}). In Gram-positive bacteria, which have only a single membrane, synthesis ends by covalent attachment of the polysaccharide to the cell wall (Figure 5).

4.1.1. Initiation of Repeat Unit Synthesis Is a Conserved Step

In the block-type mechanism, repeat unit synthesis is initiated on a lipid acceptor (Figure 4). The first sugar added to the lipid acceptor is generally

TABLE 2. Common enzymes involved in Wzy-dependent capsule formation.

E. coli enzyme	Function	*S. pneumoniae* (*S. aureus*) functional equivalents[a]
Wzi	Possible role in linkage to cell surface	None (None)
Wza	Transport across outer membrane	None (None)
Wzb	Phosphatase/kinase inhibitor	CpsB (CapC)
Wzc	Autophosphorylating tyrosine kinase	CpsC and CpsD (CapA and CapB)
WbaP/WecA	Undecaprenylphosphate hexose-1-phosphate transferase	CpsE (CapM)
Wzx	Repeat unit flippase	Cps2J (Cap5J or Cap5K)
Wzy	Repeat unit polymerase	Cps2H (Cap5J or Cap5K)
WaaL	O antigen linkage to lipid A	None (None)

[a] Number indicates serotype.

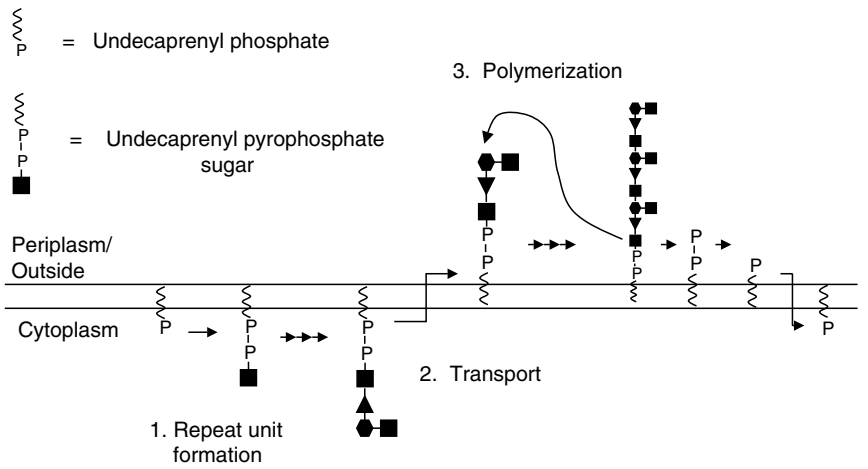

FIGURE 4. Block-type (Wzy-dependent) capsule synthesis. During polymerization, the reducing end of the maturing, long-chain polymer is transferred to a lipid-linked subunit.

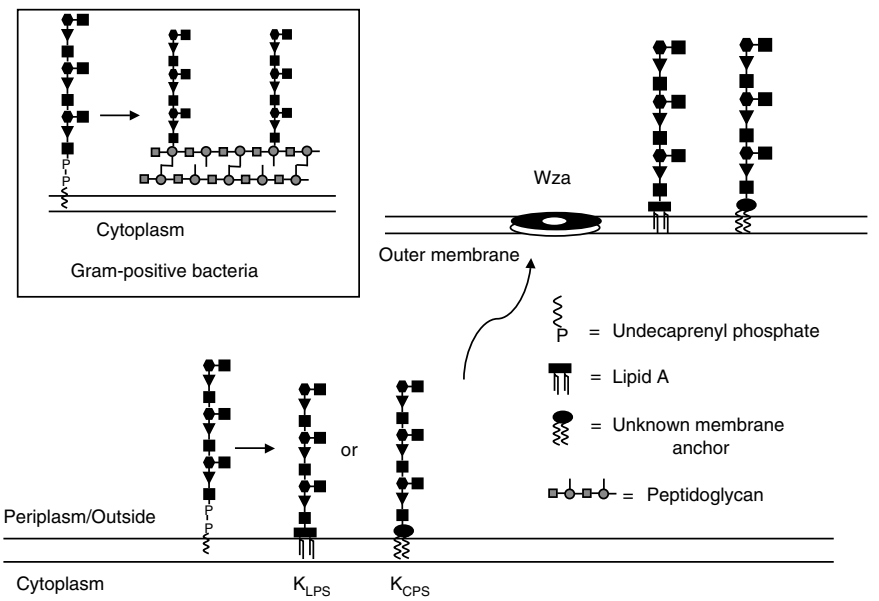

FIGURE 5. Transport and attachment of capsules in block-type (Wzy-dependent) synthesis.

Glc, Gal, GlcNAc, or GalNAc. Two enzymes have been shown to catalyze this addition in *E. coli*. WbaP, which is utilized in group 1 capsule synthesis, catalyzes the addition of either Glc or Gal, while WecA, which is utilized in

group 4 capsule synthesis, adds either GlcNAc or GalNAc (Whitfield and Roberts, 1999). WecA is also the glycosyltransferase used to initiate LPS O antigen synthesis. To date, only Glc has been shown to initiate repeat unit formation in Gram-positive bacteria. CpsE, encoded by the first gene of the serotype-specific region in streptococci, catalyzes this reaction in both *S. pneumoniae* and *S. agalactiae* (Kolkman et al., 1997a; Rubens et al., 1993; van Selm et al., 2002). In Gram-negative bacteria, and likely Gram-positive bacteria, the lipid acceptor is the isoprenoid undecaprenyl phosphate. This is the same lipid acceptor used in peptidoglycan and O antigen synthesis. In capsule synthesis, the initiating glycosyltransferase transfers a sugar-1-phosphate from a nucleotide sugar to the lipid to form an undecaprenyl pyrophosphate sugar (Whitfield and Roberts, 1999). Following this initiation of repeat unit formation, the remaining sugars of the repeat unit are added onto the nonreducing end of the growing repeat unit. Generally, each glycosidic linkage of the repeat unit is formed by a separate glycosyltransferase.

4.1.2. Transport of Capsule Repeat Units Across the Cytoplasmic Membrane

The mechanism by which the highly hydrophilic repeat unit is transported across the hydrophobic membrane is one of the more intriguing unanswered questions regarding capsule synthesis. Deletion of *wzx*, a gene involved in LPS O antigen synthesis, results in what appears to be the accumulation of O antigen repeat units on the inner face of the cytoplasmic membrane, suggesting a role for this enzyme in repeat unit transport (Liu et al., 1996). Wzx homologs can be found in capsule loci of bacteria utilizing the block-type mechanism of synthesis. Enzymes like Wzx and its functional homologs have been termed "flippases." Wzx, which contains 12 transmembrane spanning domains, shares very limited homology with putative flippases in other capsule loci (Paulsen et al., 1997). The mechanism of how flippases function is poorly understood. Studies on these enzymes have been hampered by difficulties in overexpressing the proteins and in distinguishing between the inner and outer leaflets of a membrane bilayer (Raetz and Whitfield, 2002). It is clear, however, that repeat unit transport is not dependent on the chemical structure of the repeat unit, because structures as simple as a polyprenol pyrophosphate containing a single sugar can be transported (Feldman et al., 1999; Rick et al., 2003).

4.1.3. Repeat Unit Polymerization

The mechanism for sequential linkage of repeat units has also not been well characterized due to difficulties in obtaining significant quantities of the polymerase (Raetz and Whitfield, 2002). Polymerization occurs by transfer of the reducing end of the growing polysaccharide chain onto the nonreducing end of the new repeat unit (Figure 4) (Bray and Robbins, 1967; Robbins et al., 1967). Polysaccharide growth at the reducing end of the polymer is akin to protein synthesis on the ribosome where the polypeptide chain is moved onto

the next amino acid. Polymerization of the growing polysaccharide occurs in the periplasm of Gram-negative bacteria following transport of the repeat units across the inner membrane. Likewise, capsule polymerization in Gram-positive bacteria is thought to occur following transport of the repeat units to the outer surface of the cytoplasmic membrane. Both locations lack ATP or another metabolic energy source for formation of the glycosidic linkages. The energy for the polymerization step is likely provided by the high energy retained in the phosphoryl linkages of the undecaprenyl pyrophosphate sugar.

Wzy is the apparent polymerase in *E. coli* capsule synthesis and is similar to the putative polymerase in LPS O antigen synthesis (Drummelsmith and Whitfield, 1999). Wzy, like the repeat unit transporter Wzx, contains multiple transmembrane domains and shares little homology with other putative polymerases in *E. coli* or other bacteria. Based on amino acid sequence alone, a clear distinction cannot be made between Wzy and Wzx (Raetz and Whitfield, 2002). Additionally, since polymerization is dependent on repeat unit transport, it is difficult to determine if the lack of polymerization observed in Wzy or other polymerase mutants is due to a polymerization or a transport defect. The possibility exists that both proteins may act together to perform both processes.

Following polymerization, one of the phosphates is removed from undecaprenyl pyrophosphate to regenerate undecaprenyl phosphate, which can then be recycled to the cytoplasmic face of the membrane and used for subsequent repeat unit formation. The antibiotic bacitracin, a known inhibitor of capsule and peptidoglycan synthesis, inhibits the recycling of undecaprenyl phosphate by binding irreversibly to undecaprenyl pyrophosphate (Siewert and Strominger, 1967).

In *E. coli* group 1 and group 4 capsule synthesis, polymerization is terminated by the transfer of capsule onto lipid A (in the case of K_{LPS}) or onto a lipid acceptor (in the case of K_{CPS}) (Figure 5). The enzyme responsible for transferring an undecaprenyl pyrophosphate linked capsule chain onto lipid A is WaaL (Whitfield et al., 1997), which also transfers LPS O antigen onto lipid A (Raetz and Whitfield, 2002). In Gram-positive bacteria, polymerization may be followed by transfer of the polysaccharide chains to the cell wall (Figure 5). The current information on this step in Gram-positive capsule synthesis is discussed in more detail in Section 4.1.5.

4.1.4. In Gram-negative Bacteria, the Capsule Polymer Is Transported Across the Periplasm and the Outer Membrane

Electron microscopy has shown that transport appears to occur at sites where the inner and outer membranes are in apposition, presumably by a periplasmic scaffold that links the biosynthetic machinery to an outer membrane translocation apparatus (Bayer and Thurow, 1977; Kroncke et al., 1990). These sites, termed membrane-adhesion sites or Bayer's junctions (Bayer, 1968), are, however, controversial, and there is currently very little information

on the proteins or mechanism involved. One protein known to be involved in transporting *E. coli* group 1 capsular polysaccharides across the outer membrane is Wza (Drummelsmith and Whitfield, 1999), a lipoprotein that forms a multimeric complex in the outer membrane (Figure 5) (Drummelsmith and Whitfield, 2000). Mutations in *wza* result in an impaired ability to assemble high–molecular weight K_{CPS} polymer on the cell surface but have no effect on production of the low–molecular weight K_{LPS} polymer (Drummelsmith and Whitfield, 1999). In *E. coli* group 1 capsules, *wza* is one of the four upstream common genes and is essentially identical between capsule types (Drummelsmith and Whitfield, 1999). Homologs of *wza* can also be found in the colanic acid biosynthetic locus and in the capsule loci of *K. pneumoniae* and *E. amylovora* (Drummelsmith and Whitfield, 2000). Despite the high level of homology (67% identity, 84% similarity) between Wza from the K30 capsule locus and AmsH from *E. amylovora*, AmsH does not complement defects in Wza, suggesting that these conserved proteins may recognize some specific aspect of the polysaccharide being synthesized (Drummelsmith and Whitfield, 2000). Although the exact function of Wza in capsule translocation is unknown, it has been shown to form high–molecular weight complexes that have a pore-like structure and may function similarly to secretins found in type II/III protein secretion (Drummelsmith and Whitfield, 2000) (see Chapter 9). Secretins serve as voltage-gated channels in the outer membrane through which proteins are exported. Group 4 capsules of *E. coli* do not possess a *wza* homolog in their capsule loci and the mechanism by which these capsules are transported across the periplasm and outer membrane is not known.

4.1.5. Capsules Synthesized by Gram-positive Bacteria May Be Linked to the Cell Wall

In many Gram-positive bacteria, the capsular polysaccharide is transferred onto the cell wall following polymerization (Sorensen et al., 1990). Covalent attachment of the polysaccharide to the cell wall has been observed for many capsule types in *S. pneumoniae* and *S. agalactiae*. In *S. agalactiae* type III, the capsule is linked to the GlcNAc residue of peptidoglycan via a phosphodiester bond and an oligosaccharide linker (Deng et al., 2000). The enzyme(s) and specific requirements for completing the transfer are not known, but in *S. pneumoniae*, the transfer of capsule chains to the cell wall can occur independent of polymer size (Bender et al., 2003).

4.1.6. The Upstream Common Genes in the Capsule Loci of *E. coli* Group 1 Capsules and Gram-positive Capsule Loci Are Involved in Modulation of Capsular Chain Length and Transport

In *E. coli*, the upstream common genes are *wzi*, *wza*, *wzb*, and *wzc* (Figure 1). The function of Wzi is unknown, but it has been proposed to play a role in linking the high–molecular weight capsule to the cell surface (Rahn et al.,

2003). Wza, as described above, forms a pore-like structure in the outer membrane and is involved in translocation of the capsule to the outer surface of the outer membrane. Wzc is a homolog of the *E. coli* Wzz and the *Sinorhizobium meliloti* ExoP, which are involved in chain length control of the LPS O antigen and the EPS succinoglycan, respectively (Becker et al., 1995; Drummelsmith and Whitfield, 1999). Mutations in *wzz*, *exoP*, and wzc_{K30} result in changes in chain length with a reduction in high–molecular weight polymer. In addition, wzc_{K30} mutations result in a change in the banding pattern of the K_{LPS} (Drummelsmith and Whitfield, 1999). The structures of Wzc, Wzz, and ExoP are similar, with each containing a periplasmic domain flanked by transmembrane domains. Wzc and ExoP, however, possess an additional C-terminal cytoplasmic domain that contains a consensus ATP-binding site, and these proteins exhibit autophosphorylating tyrosine kinase activity (Niemeyer and Becker, 2001; Vincent et al., 1999; Wugeditsch et al., 2001). Wzb is a low–molecular weight acid phosphatase that can dephosphorylate Wzc in vitro (Vincent et al., 1999; Wugeditsch et al., 2001). Phosphorylation of Wzc_{K30} correlates positively with capsule production (Wugeditsch et al., 2001), whereas phosphorylation of Wzc_{CA}, the Wzc homolog in colanic acid biosynthesis, appears to negatively influence colanic acid synthesis (Vincent et al., 2000).

Wzc homologs also occur in Gram-positive bacteria where, as in *E. coli* group 1 capsules, they are encoded by genes located in the upstream common region of the capsule loci (Figure 3). For *S. pneumoniae*, the genes *cpsA*, *cpsB*, *cpsC*, and *cpsD* comprise this region. CpsA shares homology with LytR, a transcriptional attenuator of autolytic activity in *Bacillus subtilis*. In the streptococci, the exact function of CpsA is unknown, but decreases in capsule transcript levels following deletion of the *cpsA* homolog in *S. agalactiae* suggest a possible role in regulation (Cieslewicz et al., 2001). CpsC and CpsD share homology with the N and C termini, respectively, of Wzc as well as ExoP. CpsD is an autophosphorylating tyrosine kinase that requires CpsC for its initial phosphorylation (Bender and Yother, 2001; Morona et al., 2000). CpsB is a phosphotyrosine phosphatase that may also act as a kinase inhibitor (Bender and Yother, 2001), but unlike the *E. coli* phosphatase, it lacks homology with low–molecular weight acid phosphatases. Like $Wzc_{K30,}$ phosphorylation levels of CpsD correlate positively with capsule production, and mutations in *cpsC* or *cpsD* result in reductions in chain length (Bender et al., 2003). In this system, additional components may be essential for modulation, as a positive correlation between CpsD phosphorylation and capsule production is not observed in all genetic backgrounds (Bender et al., 2003).

The mechanisms by which phosphorylation of Wzc and its homologs modulate capsule production and chain length have not been determined. Possible affected steps include transport, polymerization, and repeat unit formation (Gonzales et al., 1998). Regulation of capsule production at the level of repeat unit formation rather than polymerization would prevent the unnecessary formation of repeat units and conserve nucleotide sugars and

lipid acceptors that could be used for other cellular functions. In vitro, Wzc_{CA} can transphosphorylate Ugd, a UDP-Glc dehydrogenase necessary for formation of colanic acid repeat units, and modulate its activity (Grangeasse et al., 2003). Although it is attractive to speculate that control of repeat unit formation, or another step, occurs by transphosphorylation and modulation of enzyme activity, it is also possible that phosphorylated Wzc/CpsD has an indirect effect through an intermediate factor. The presence or absence of this intermediate would explain why phosphorylation of CpsD or Wzc can have opposite effects on polysaccharide production depending on the polymer or genetic background under study.

4.1.7. The Rcs Two-component Regulatory System Is Involved in Regulating Colanic Acid and Group 1 Capsule Biosynthesis *in E. coli*

The Rcs two-component regulatory system is composed of the transmembrane sensor kinase, RcsC; the response regulator, RcsB; and an accessory factor, RcsA (Gottesman, 1995). Binding of phosphorylated RcsB to the promoter region of the colanic acid biosynthetic locus increases transcription, and this effect is enhanced by *interaction* of RcsA with RcsB. Colanic acid levels are reduced above 30°C due to degradation of RcsA by an active Lon protease. Homologs of the Rcs system can be found in *E. coli* and other Gram-negative bacteria expressing group 1 or group 1-like capsules. *E. coli* group 1 strains that contain mutations in *rcsA* or *rcsB* are limited in their ability to produce high levels of capsule, although not to the extent observed with colanic acid. The differing effects may be explained by differences in the sequences of RcsB binding sites located upstream of the group 1 capsule and colanic acid promoters (Whitfield and Roberts, 1999).

4.2. ABC-2 Transporter-dependent Pathway

Unlike the Wzy-dependent pathway, most of the polymers synthesized by the ABC-2 transporter-dependent mechanism are relatively simple, being composed of either a single monosaccharide or a heterodisaccharide repeat. This pathway, so far, has been observed only in Gram-negative bacteria, and is the pathway used to synthesize *E. coli* group 2 and 3 capsules, as well as the capsules of *Neisseria* and *Haemophilus* Spp. The majority of *E. coli* isolates that are associated with extraintestinal disease synthesize capsules by this mechanism. Some of the better-described examples in this group are the *E. coli* K1, K4, and K5 capsules. In contrast to the Wzy-dependent pathway, these polymers are synthesized in the cytoplasm and then transported across the inner membrane by a dedicated, conserved ABC-2 transporter (Figure 6). The synthesis and transport enzymes form hetero-oligomeric protein complexes (Rigg et al., 1998). The functions of proteins common to this mechanism of synthesis are described in Table 3.

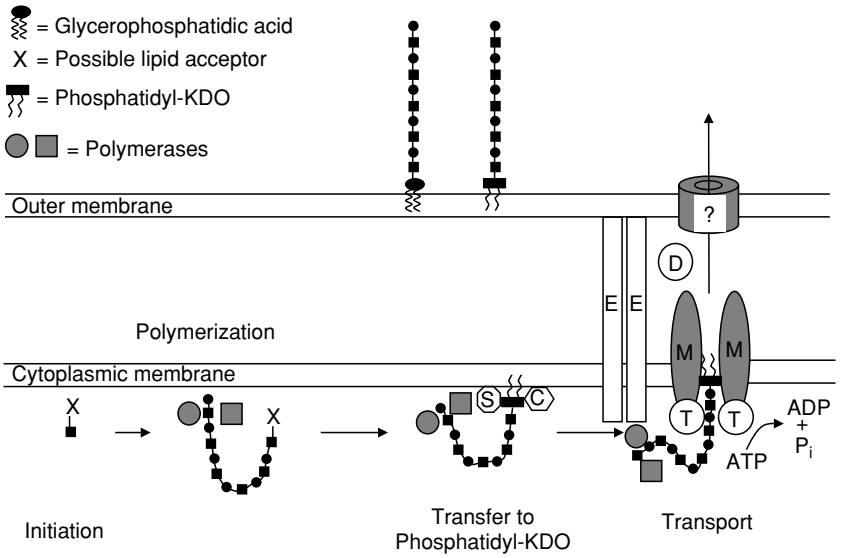

FIGURE 6. ABC-2 transporter-dependent capsule synthesis. Capsules are anchored in the outer membrane by either phosphatidyl-KDO or glycerophosphatidic acid. (Pathway derived from Arrecubieta et al., 2001; Bliss and Silver, 1996; Rigg et al., 1998; Whitfield and Roberts, 1999.)

TABLE 3. Common enzymes involved in ABC-2 transporter-dependent capsule synthesis.

Enzyme	Function
KpsM	Transmembrane portion of ABC-2 transporter
KpsT	ATPase portion of ABC-2 transporter
KpsC	Involved in addition of phosphatidyl-KDO to polymer
KpsS	Involved in addition of phosphatidyl-KDO to polymer
KpsU	CMP-KDO synthetase
KpsE	Transmembrane protein involved in transperiplasmic export
KpsD	Periplasmic protein involved in transperiplasmic export

4.2.1. ABC-2-transporter-dependent Capsular Polysaccharides are Synthesized by a Processive Mechanism

Unlike the Wzy-dependent polymers, the repeating structures of ABC-2-transporter dependent polymers are not built upon a lipid acceptor and then polymerized. Instead, these polymers are synthesized in the cytoplasm by either a single glycosyltransferase or by the concerted efforts of several glycosyltransferases (Figure 6). The addition of sugars occurs at the nonreducing end of the polymer by a processive mechanism, whereby the enzymes complete synthesis of one chain before initiating synthesis of another. For capsule types K4 and K1, only a single enzyme is required for the polymerization (Ninomiya et al., 2002; Steenbergen et al., 1992). These enzymes are

similar to those used for the synthase-dependent pathway described below, in that they are able to form multiple glycosidic linkages. For the K5 capsule, two enzymes working in tandem are involved (Hodson et al., 2000). Despite similarities to the mechanism used for synthesis of the polymannan-containing LPS O antigens O8 and O9, where initiation involves WecA-catalyzed initiation on an undecaprenyl pyrophosphate-linked GlcNAc (Raetz and Whitfield, 2002), a requirement for an undecaprenyl-linked sugar intermediate has not been described and WecA is not required.

4.2.2. Transport of the Polymer Requires Addition of a Lipid Moiety and an ABC-2 Transporter

At some point during polymerization, the lipid phosphatidyl-3-deoxy-manno-2-octulosonic acid (phosphatidyl-KDO) is added to the reducing end of the polysaccharide chain. The addition of phosphatidyl-KDO is thought to be catalyzed by the action of two enzymes, KpsC and KpsS (Bronner et al., 1993), encoded by region 1 genes (Figure 6). Phosphatidyl-KDO is synthesized by using the substrate CMP-KDO, which in group 2 capsules is synthesized in part by KpsU (Pazzani et al., 1993). The precise point at which phosphatidyl-KDO is added is unclear, although it has been suggested that addition follows polymerization and is a signal for termination and export (Whitfield and Roberts, 1999). Phosphatidyl-KDO also serves to anchor the polymer to the outer membrane. However, some group 2 capsules, such as the K1 and K92 polysialic polymers, do not add phosphatidyl-KDO but rather use a glycerophosphatidic acid to anchor the polysaccharide.

Transport across the membrane involves an ABC-2 transporter, which is composed of KpsM, the integral membrane component, and KpsT, which contains the ATPase activity (Bliss and Silver, 1996). Both of these proteins are encoded in region 3 of the capsule loci. Two proteins encoded in region 1 are also involved in transporting the polysaccharide chain across the periplasm to the outer membrane (Arrecubieta et al., 2001; Bliss and Silver, 1996). KpsE is an integral cytoplasmic membrane protein, and KpsD is predominately a soluble periplasmic protein that can also be found associated with the inner and outer membrane fractions. The current model for KpsD function is that it transiently moves from the inner membrane to the outer membrane, interacting with the polysaccharide to transit it across the periplasm. KpsE, which appears to form dimers in the membrane, may function to connect the two membranes and facilitate this process. Since capsular polysaccharide is required for correct KpsD localization, the polysaccharide itself may function to link all these proteins together to form the transperiplasmic transport apparatus (Arrecubieta et al., 2001). How the polymer transits the outer membrane is not known. Unlike group 1 capsules, there is not a Wza pore-like molecule encoded in the group 2 or group 3 capsule loci. Additionally, the outer membrane proteins OmpT, LamB, OmpF, OmpA, and OmpC do not play a role in K5 capsule expression on the cell surface (Arrecubieta et al., 2001).

4.2.3. Regulation of Group 2 Capsules is Very Complex

Each of the three segments of the K5 locus has a separate mechanism of regulation. The six region 1 genes (*kpsFEDUCS*) are transcribed from a single promoter upstream of *kpsF* (Figure 2). However, the transcript is processed to generate a separate transcript for *kpsS*, which encodes one of the enzymes necessary for attachment of phosphatidyl-KDO (Simpson et al., 1996). By regulating KpsS levels, entry of polysaccharide chains into the transport machinery may be controlled. The region 1 and 3 promoters are thermoregulated, with no detectable transcription from either occurring at temperatures below 20˚C (Simpson et al., 1996; Stevens et al., 1997).

Upstream of the region 3 promoter of the group 2 capsule locus is a conserved 39 bp motif termed the JUMPstart (*j*ust *u*pstream of *m*any *p*olysaccharide-associated gene *start*s) sequence. This sequence occurs upstream of many Gram-negative polysaccharide biosynthetic operons, including the LPS core (*rfa*) operon, the LPS O antigen (*rfb*) operon, and the colanic acid (*cps*) biosynthetic locus. The most conserved part of the JUMPstart sequence is an 8 bp sequence (5′-GGCGGTAG-3′) termed the operon polarity suppressor (OPS) element. It is believed that the ops element helps recruit the transcriptional regulator RfaH to the transcription complex, where it acts as a processivity factor for RNA polymerase and increases expression of genes distal to the promoter (Bailey et al., 1997). RfaH is required for K5 capsule production and allows for sufficient transcription of the region 2 genes via the promoter and JUMPstart sequence upstream of the region 3 genes (Stevens et al., 1997). Region 2 contains several promoters of its own; however, these do not result in enough transcription for capsule production.

4.3. Synthase-dependent Pathway

The synthase-dependent pathway is used to produce only a few capsules, each of which is composed of relatively simple polysaccharides. These polysaccharides, like those synthesized by the ABC-2 transporter-dependent pathway, comprise either a monosaccharide or a heterodisaccharide repeat and require only a single glycosyltransferase (the synthase) to form all the glycosidic linkages. No additional enzymes or proteins are necessary to transport the polysaccharide, however, as the synthase catalyzes the formation of the glycosidic linkages and simultaneously transports the growing polymer across the cytoplasmic membrane (Figure 7). Synthases belong to a family of processive β-glycosyltransferases whose members include chitin synthases, cellulose synthases from bacteria and plants, the Nod factor synthase from *Rhizobium*, and eukaryotic HA synthases. Two well-studied capsules that utilize this pathway are the type 3 capsule [-3)-β-D-GlcUA-(1–4)-β-D-Glc-(1-] of *S. pneumoniae* and HA [-4)-β-D-GlcUA-(13)-β-D-GlcNAc-(1-] of *S. pyogenes* and *Pasteurella multocida*, which are described below.

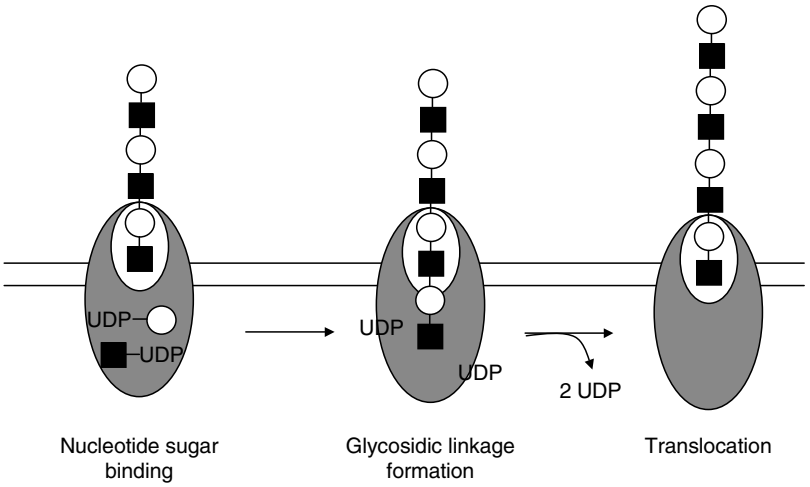

FIGURE 7. Synthase-dependent capsule synthesis. The synthase, located in the cytoplasmic membrane, contains binding sites for UDP sugars and the growing polysaccharide chain.

4.3.1. The Model for Synthase Function Comes from Analyses of Their Secondary Structures and the Structures of Their Polysaccharides

The enzymes in the processive β-glycosyltransferase family do not share a high level of amino acid similarity but do have a conserved secondary structure. Hydrophobic cluster analyses (HCA) of these proteins have revealed two conserved domains (Keenleyside and Whitfield, 1997; Saxena et al., 1995). Domain A is found in almost all glycosyltransferases, while domain B is unique to the processive β-glycosyltransferases family. Processive β-glycosyltransferases were originally proposed to contain two nucleotide sugar-binding sites, as well as a site for the growing polysaccharide chain, and to grow by dual addition of sugars (Saxena et al., 1995). HA, type 3 polysaccharide, cellulose, and other polymers containing alternating β-glycosidic linkages exhibit a twofold symmetry in which each sugar is oriented approximately 180 degrees relative to the adjacent sugar (Gardner and Blackwell, 1974; Heatley and Scott, 1988; Minke and Blackwell, 1978). Saxena et. al (1995) proposed that the binding sites for the two nucleotide sugars would be diametrically opposed, allowing for the generation of polymers with a twofold symmetry to be generated without having to twist the polymer or the enzyme 180 degrees after the addition of each sugar. Synthesis of type 3 polysaccharide in *S. pneumoniae* is proposed to occur in a three-step process, essentially as shown in Figure 7. First, the nucleotide sugars bind to their respective binding sites followed by sequential glycosidic linkage formation. Hydrolysis of the nucleotide sugars and release of two UDP molecules presumably provide the energy to translocate the polymer through the polysaccharide

binding site and the membrane. Although the enzymes from this class were originally thought to grow from the reducing end (Saxena et al., 1995), recent data on the type 3 synthase from *S. pneumoniae* (Cartee et al., 2000), the cellulose synthase from *Cladaphora* and *Acetobacter* (Koyama et al., 1997), and the *P. multocida* HA synthase (pmHAS) (DeAngelis, 1999), as well as the original characterization of the *S. pyogenes* HA synthase (spHAS) (Stoolmiller and Dorfman, 1969), have shown that growth occurs from the nonreducing end. Conflicting data indicating reducing end growth, however, still exists for the synthesis of HA in eukaryotic cells (Asplund et al., 1998; Prehm, 1983a, b).

Recently, generation of a crystal structure for one member of the processive β-glycosyltransferase family led to the prediction that these enzymes contain only a single nucleotide sugar binding site (Charnock et al., 2001). Charnock et al. have suggested that the second nucleotide sugar binding site for polymers composed of a heterodisaccharide repeat, like type 3 polysaccharide and HA, would be generated by the addition of the opposite sugar to the polymer (Charnock et al., 2001). In other words, the two binding sites are separated temporally rather than spatially. Confirmation of this model, however, awaits experimental evidence and/or the crystal structure of an enzyme that catalyzes two distinct glycosidic linkages.

All processive β-glycosyltransferases are integral membrane proteins containing 2–6 predicted transmembrane domains. Generally, enzymes that catalyze the export of large molecules like polysaccharides and proteins have at least 12 transmembrane domains (Paulsen et al., 1997), suggesting that enzymes like type 3 synthase and HASs may function as multimers in order to transport their polymers across the membrane. Studies on the spHAS and *S. equisimilis* (seHAS), however, have shown that these enzymes function as monomers (Tlapak-Simmons et al., 1998). Both enzymes interact tightly with 16 molecules of cardiolipin that could potentially form a pore through which the growing polysaccharide is extruded (Tlapak-Simmons et al., 1998).

4.3.2. The Mechanisms Controlling Initiation and Regulation of Synthesis may Vary Among Synthase-dependent Polymers

Although the mechanism of polymerization for polymers like type 3 polysaccharide and HA has been well characterized, there is very little information on how initiation of synthesis occurs. HA synthesis appears to occur de novo from the nucleotide sugars, without a requirement for a lipid intermediate (Saxena et al., 1995). The *S. pneumoniae* type 3 synthase, however, utilizes a glycerophosphate lipid acceptor to initiate synthesis (Cartee et al., 2001), and the related cellulose synthase CesA from *Arabidopsis* uses β-sitosterol-β-glucoside as an acceptor (Peng et al., 2002).

Control of polymer formation may occur at the level of transcription or substrate concentration. For the *S. pneumoniae* type 3 synthase and the eukaryotic and *S. pyogenes* HA synthases, premature release of the nascent

polysaccharide chain from the enzyme during in vitro biosynthetic reactions occurs when one of the substrates becomes limiting or is omitted from the reaction (Forsee et al., 2000; Prehm, 1983a; Sugahara et al., 1979). In *S. pneumoniae*, this premature release has been demonstrated to be an enzymatic mechanism (Forsee et al., 2000). The current model suggests that binding of a single nucleotide sugar may trigger the premature translocation of the polymer through the polysaccharide-binding site. In the absence of extension of the polysaccharide chain by two additional sugars, the enzyme cannot recognize the terminal nonreducing end, and the polymer is released. The concentration of nucleotide sugars in the cell may thus be very important for determining whether polymer synthesis or chain termination and release occur. Some nucleotide sugars, such as UDP-GlcUA for the *S. pneumoniae* type 3 and *S. pyogenes* HA capsules, are used only for capsule synthesis, and their concentrations may be important points for control.

In *S. pyogenes*, control of HA synthesis also occurs at the transcriptional level. In laboratory culture, loss of capsule production occurs during stationary phase and correlates with a loss of both biosynthetic activity and transcription of the *has* operon (Crater and van de Rijn, 1995; van de Rijn, 1983). A two-component regulatory system, alternately designated CsrR/S CsrS or CovR/CovS, is also involved in negative regulation of HA capsule gene expression (Bernish and van de Rijn, 1999; Levin and Wessels, 1998).

5. Nonpolysaccharide Capsules

Although polysaccharide capsules are by far the most common type produced by bacteria, antiphagocytic polypeptide capsules have been described for some *Yersinia* and *Bacillus* spp. (Du et al., 2002; Makino et al., 1989). γ-polyglutamic acid (PGA) was first described as part of the extracellular material produced by *B. anthracis*, the bacterium responsible for causing anthrax, and has now been identified in *B. subtilis* as well as several other *Bacillus* spp. The genes required for PGA synthesis are located on the 96 kb plasmid pXO2 in the operon *capBCA*. Positive control of capsule gene expression is mediated by *acpA* and *atxA*, which are located on pXO2 and 182 kb pXO1, respectively (Koehler, 2002). From studies of the homologous enzymes in *B. subtilis*, *capB* likely encodes the γ-polyglutamate synthetase, whereas CapC may be involved in transport (Urushibata et al., 2002). Also encoded on pXO2 is Dep, which is associated with depolymerization of PGA and may be involved in controlling the size of the polymer (Uchida et al., 1993).

Proteins required for synthesis of the *Yersinia pestis* fraction 1 (F-1) capsule antigen are encoded by four genes—*caf1*, *caf1M*, *calf1A*, and *caf1R*—located on the 100 kb plasmid pFra, The polypeptide capsule consists of 15 kDa subunits encoded by *caf1* and assembled through the actions of a chaperone (Caf1M) and an outer membrane usher (Caf1A) (Galyov et al., 1990, 1991; Karlyshev et al., 1992b). Caf1R acts as a positive transcriptional regulator (Karlyshev et al., 1992a).

6. Conclusions

Capsules play vital and varied roles in the pathogenesis of bacteria. Despite the enormous variability in their structures and the diversity of organisms in which they occur, striking similarities are found in both their genetics and mechanisms of synthesis. In many cases, the similarities can be extended to eukaryotic polysaccharides, allowing the models derived from studies of bacterial capsules to serve as paradigms for a broad spectrum of organisms. Over the last decade, the genetic characterization of numerous bacterial capsule loci has led to a renewed interest in the field and has provided the tools to help dissect the complicated pathways involved in polysaccharide synthesis and regulation. In turn, the mechanisms by which capsules function in pathogenesis are now being more fully explored and appreciated.

Questions to Consider

1. Why do bacteria regulate capsule expression and production?

Bacteria regulate capsule expression and production to fulfill different functions depending on the environment they occupy. For instance, bacteria that colonize the nasopharynx may turn down capsule production to expose adherence factors important for the colonization event, but would increase capsule production when in the bloodstream in order to protect against opsonophagocytosis. Reducing capsule synthesis when it is not essential also reduces metabolic drain on the cell, allowing nucleotides, sugars, lipids, and other precursors to be used for other functions.

2. What criteria are used to distinguish capsule groups in E. coli?

E. coli capsules can be divided into four groups based on genetic and biochemical criteria including: the location of the capsule locus on the chromosome, the organization of the capsule locus, the mechanism of synthesis utilized, whether CMP-KDO synthesis is elevated, whether other cell-surface polysaccharides are produced, and whether capsule production is temperature-regulated.

3. What are the similarities between group 1 capsules of *E. coli* and the majority of capsules in Gram-positive bacteria?

The *E. coli* group 1 capsule loci and the Gram-positive capsule loci share several common features. Both loci are transcribed via a single promoter and contain a type-specific region that encodes the enzymes necessary to synthesize a particular capsule type and flanking common regions that occur in all capsule types. The upstream common regions of both *E. coli* and Gram-positive bacteria encode proteins involved in control of capsule levels.

4. What mechanisms do bacteria use to transport capsules?

Three different mechanisms of capsule and repeat unit transport have been described. For polymers built by the block-type mechanism, the repeat units

are transported across the cytoplasmic membrane via a flippase where they are subsequently polymerized. Capsules synthesized by the ABC-transporter mechanism are polymerized in the cytoplasm and are subsequently transported via an ABC transporter. In the synthase-dependent pathway, the capsule chains are synthesized and exported across the membrane via the synthase.

5. How do capsules contribute to the virulence of pathogenic bacteria?

Capsules can contribute to the virulence of bacteria in several ways. The most common means is by inhibiting opsonophagocytosis. Capsules can serve to prevent either the deposition of complement on the bacterial surface or the recognition of complement bound to the surface by host phagocytic receptors. In Gram-negative bacteria, capsules may also prevent complement-mediated lysis by preventing formation or insertion of the membrane attack complex. In addition, capsules can play a role in adherence, in the formation of biofilms, abscess formation, and in protection from desiccation.

6. What other cellular pathways are involved in, or related to, capsule synthesis?

The production of capsules requires the use of both lipids and nucleotide sugars that are used in the synthesis of other cell surface polymers. For instance, in the block-type mechanism, the repeat units are assembled on an undecaprenyl phosphate, which is the same lipid acceptor used in the formation of peptidoglycan, ECA, O antigen, and teichoic acids. Synthesis of *E. coli* group 1 and group 4 capsules also uses enzymes and lipids involved in lipopolysaccharide production. In addition, the formation of capsules and each of these polymers may require many of the same nucleotide sugars.

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Chapter 6

Bacterial Cell Walls

JENNIFER K. WOLF AND JOANNA B. GOLDBERG

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Historical Landmarks

1884 Robert Koch puts forth his postulates for infection transmissibility (Koch, 1884).

Hans Christian Gram develops the Gram stain (Gram, 1884).

1885 Paul Ehrlich espouses theories of chemical toxicity on bacteria (Ehrlich, 1885).

1891 Paul Ehrlich proposes that antibodies are responsible for immunity (Ehrlich, 1891).

1894 Richard Pfeiffer discovers endotoxin in *Vibrio cholerae* (Pfeiffer, 1894).

1923 Michael Heidelberg and O.A. Avery show that bacterial carbohydrates can be virulence agents (Heidelberger and Avery, 1923).

1928 Frederick Griffith discovers the properties of transformation between bacteria, when unencapsulated, avirulent *Streptococcus pneumoniae* are transformed into encapsulated, virulent strains when exposed to heat-killed virulent strains (Griffith, 1928).

1929 Alexander Fleming discovers penicillin (Fleming, 1929).

1931 Margaret Pittman identifies variations, such as encapsulation, that can be used for identification and determination of the influence on pathogenicity (Pittman, 1931).

1987 *Haemophilus influenzae* type b (Hib) vaccine, composed of a bacterial polysaccharide–protein conjugate, licensed (Schneerson et al., 1984).

1988 Vancomycin-resistant strain of *Enterococcus faecium* isolated (Facklam et al., 1989).

1997 First human Toll-like receptor identified (Medzhitov et al., 1997).

Staphylococcus aureus strain with decreased vancomycin susceptibility isolated (Visalli et al., 1998).

1. Introduction

The cell wall surrounds the bacterium and can protect it from the environment. In the case of pathogenic bacteria, the nature of the cell wall dictates much about its interactions with the host's innate immune system and in some cases the diseases it can cause, as well as susceptibility or resistance to antibiotics. The structures embedded within the cell wall can also be crucial to cell growth, environmental regulation, adherence, and virulence.

2. The Gram Stain

Bacteria have been historically classified based on shape and cell wall structure, which can be revealed through a powerful and simple technique called

the Gram stain. Gram-negative and Gram-positive bacteria show differential staining when using the Gram stain technique, due to the differences in composition of the cell wall also called the cell envelope. These differences in cell wall structure can be detected upon microscopic observation. However, some bacteria do not show these typical reactions in the Gram stain due to differences in the cell wall structure; the lack of reaction is a defining characteristic of these bacteria. It is also important to note that the underlying structure of the cell wall determines the shape of the bacterium, although both Gram-positive and Gram-negative bacteria can have similar morphology. Typically, bacterial cells either resemble spheres (cocci) or rods (bacilli); however, other shapes can also be found (Figure 1).

Discovered by Hans Christian Gram in 1844, the Gram stain distinguishes between two different classes of bacteria: Gram-positive and Gram-negative. In the first step of the Gram stain, a sample of bacteria is heat-fixed onto a glass slide. The slide is then stained with crystal violet. After rinsing with water, iodine is added. Iodine forms a complex with the crystal violet dye that lodges in the peptidoglycan layer of both Gram-positive and Gram-negative bacteria. After another wash step, an alcohol-based decolorizer is added which is unable to elute the dye–iodine complex from the cell wall in Gram-positive bacteria. However, the thinner peptidoglycan layer in Gram-negative bacteria does not retain the crystal violet–iodine complex, and the dye washes out. At this stage, Gram-positive bacteria remain purple in appearance, whereas Gram-negative bacteria become clear. The sample is counterstained with safranin, to stain the uncolored Gram-negative bacteria red (Figure 2).

While the Gram stain is a powerful tool for classifying most bacteria, there are a few genera that do not fall into the category of Gram-positive or Gram-negative. Although their structure more closely resembles that of a Gram-positive, the acid-fast bacteria, such as the Mycobacterium, do not stain due to the presence of a waxy layer on the outside of the cell wall. The other exception to this Gram stain test is the *Mycoplasma*, which are also undetectable by Gram stain, because they have no rigid cell wall.

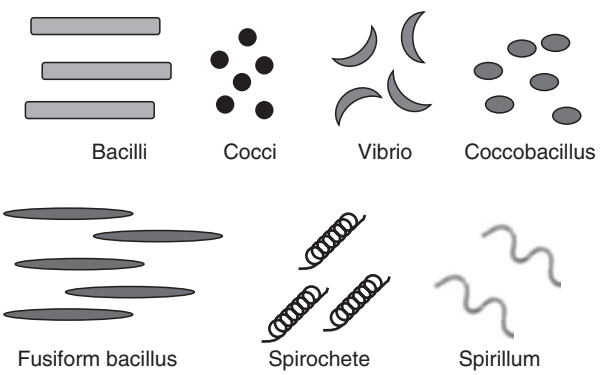


FIGURE 1. The shapes of bacteria.

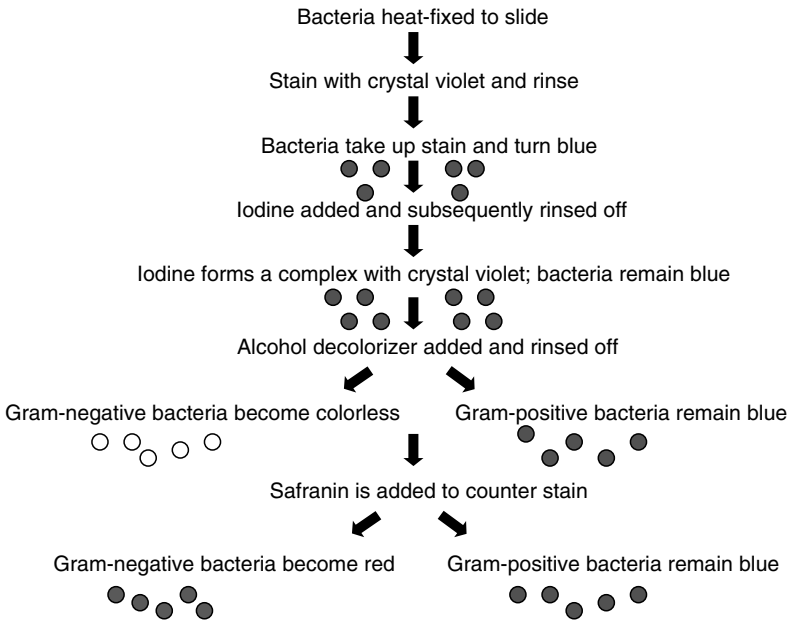

Bacteria heat-fixed to slide
Stain with crystal violet and rinse
Bacteria take up stain and turn blue
Iodine added and subsequently rinsed off
Iodine forms a complex with crystal violet; bacteria remain blue
Alcohol decolorizer added and rinsed off
Gram-negative bacteria become colorless
Gram-positive bacteria remain blue
Safranin is added to counter stain
Gram-negative bacteria become red
Gram-positive bacteria remain blue

FIGURE 2. The Gram stain and how it works.

3. Gram-positive Bacteria

In Gram-positive bacteria, the cell wall lies atop the inner membrane and protects the bacteria from lysis during changes in osmolarity (Figure 3). The Gram-positive cell wall comprises a thick (20–50 μm) layer of peptidoglycan (also referred to as murein), which can be as much as 90% of the dry weight of the wall. Peptidogylcan is composed of a repeating glycan chain of two sugars (*N*-acetyl glucosamine and *N*-acetyl muramic acid) and a tetrapeptide

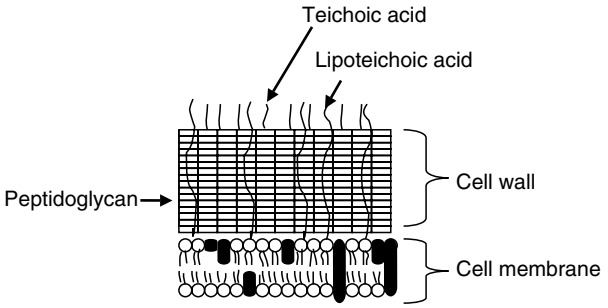


FIGURE 3. Gram-positive cell wall. Lipoteichoic acid anchors the murein wall to the cytoplasmic membrane.

attached to the *N*-acetyl muramic acid. The tetrapeptide chains are cross-linked by peptide bonds between the terminal amino acid of one subunit and the penultimate amino acid of another, to form a dense layer of peptidoglycan (Figure 4A).

Synthesis of individual peptidoglycan subunits follows the Embden Meyerhof pathway, in which fructose-6-phosphate is converted to UDP-*N*-acetyl glucosamine and UDP-*N*-acetyl muramic acid to form the base constituent of the peptidoglycan precursor (Figure 4B). Individual amino acids are added in a species-specific order to this precursor, with the exception of D-alanyl-D-alanine, which completes the formation of the peptidoglycan precursor.

The structure of the individual peptidoglycan subunit varies substantially across genera. Prior to incorporation into the cell wall, pentapeptides usually comprise D-alanine, D-alanine, a diamino acid (such as lysine or ornithine), D-glutamic acid, L-alanine, linked to *N*-acetyl muramic acid, which is in turn linked to *N*-acetyl glucosamine, and a lipid carrier. During wall synthesis, the pentapeptide is cleaved at the second D-alanine to be linked to another tetrapeptide. Linkage between these tetrapeptides varies according to genera. For example, in the Gram-positive pathogen *Staphylococcus aureus*, tetrapeptides are linked via a pentaglycine bridge (Schneider et al., 2004) (Figure 5A), while other Gram-positive bacteria have different bridges, and Gram-negative bacteria generally have direct linkages (Figure 5B).

Peptidoglycan is incorporated into the cell wall along the axis of growth, although in rods this also occurs along the whole surface and at the septum during division. As the bacterium grows, enzymes called autolysins are upregulated and dissolve the linkage between patches of the cell wall, allowing the peptidogycan tetrapeptides to separate from one another as the bacterium elongates. New peptidoglycan pentapeptides are cleaved and inserted into these patches, and linked into the macromolecule by transpeptidases (also known as penicillin-binding proteins). This process facilitates the linkage of one tetrapeptide with the terminal D-alanine of the other tetrapeptide (Popham and Young, 2003). Peptidoglycan itself can have toxic effects on host cells. In fact, tracheal cytotoxin from the Gram-negative bacteria *Bordetella pertussis*, which has been shown to kill ciliated cells, is actually a peptidoglycan fragment that is released into the extracellular fluid as a result of cell lysis or membrane turnover (Luker et al., 1995).

Teichoic acid is found uniquely in the cell wall of Gram-positive bacteria, where it binds to peptidoglycan polymers via phosphodiester bridges (Figure 3). Teichoic acid constitutes 10–50% of the cell wall. The primary structure consists of a polyribitol phosphate head, which becomes covalently linked to the muramic acid of the peptidoglycan during wall synthesis.

Lipoteichoic acid, a lipid-linked form of teichoic acid, is an amphiphile. The hydrophilic domains of these molecules are exposed on the surface of the cell wall, while the lipid tails anchor lipoteichoic acids and the cell wall firmly to the cytoplasmic membrane (Figure 3). In addition to its properties as a

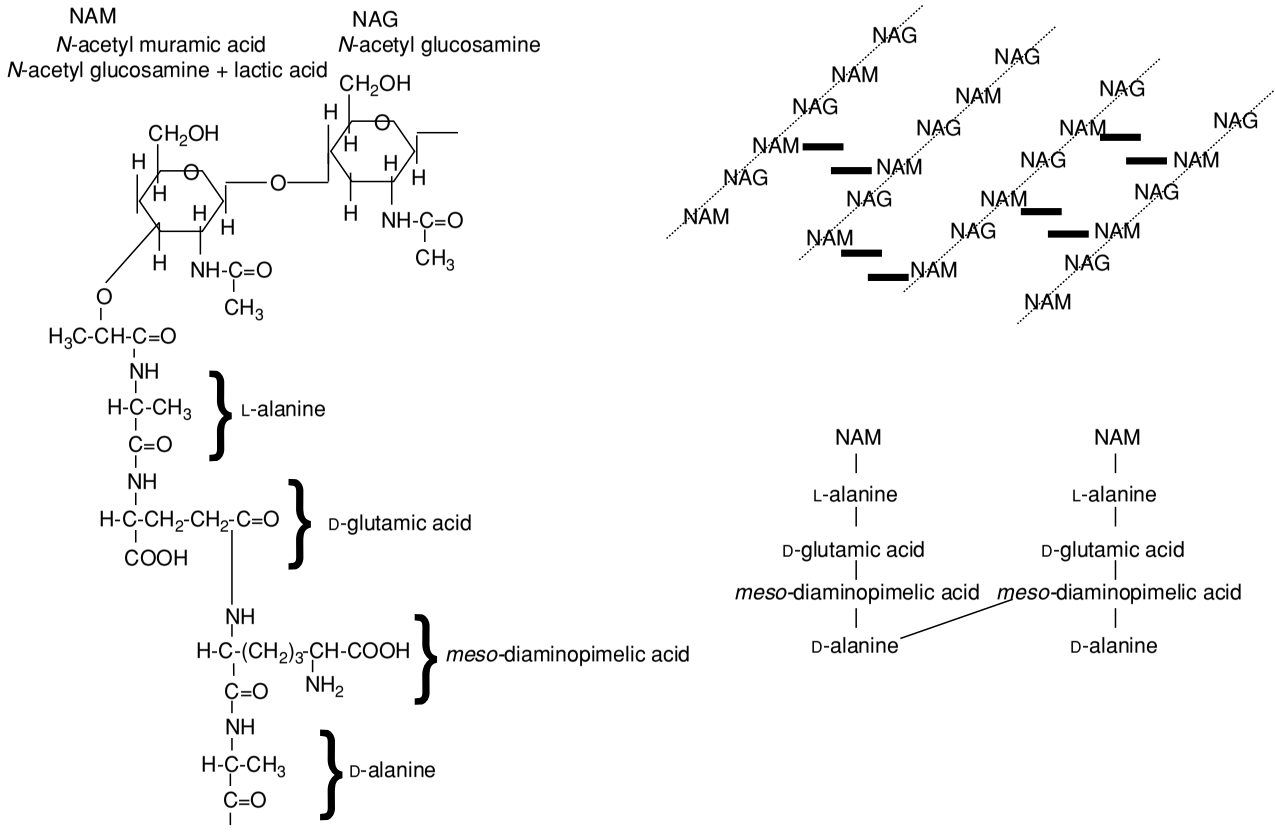


FIGURE 4. (A) Structure of *E. coli* peptidoglycan. L-alanine, D-glutamic acid, meso-diaminopimelic acid, and D-alanine are examples of the peptides represented in (B) as light and black circles.

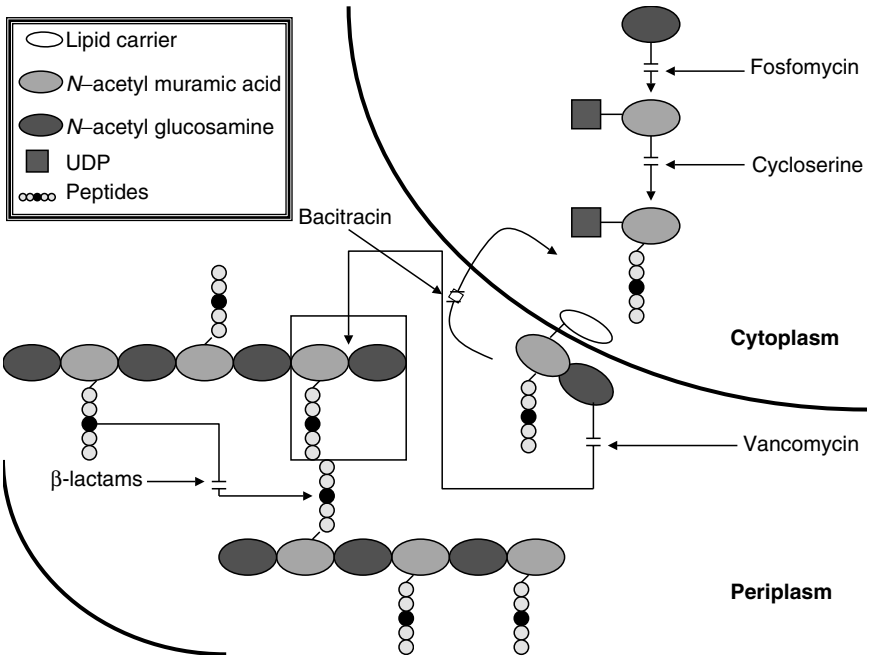


FIGURE 4. (*Cont'd*). (B) Peptidoglycan synthesis and antibiotics that target the cell wall.

surface antigen, lipoteichoic acid has also been shown to function as an adhesin in several species such as *Streptococcus pneumoniae* (McCloskey et al., 1993; Palaniyar et al., 2002).

During infection, components of the Gram-positive cell wall, including lipoteichoic acid, teichoic acid, and peptidoglycan are shed into surrounding media and host tissues, and can induce an immune response from the host similar to that elicited by lipopolysaccharide (LPS) (Ginsburg, 2002).

4. Gram-negative Bacteria

The cell wall of Gram-negative bacteria is more complex than that of Gram-positives, having an outer membrane that covers the surface of the peptidoglycan, resulting in the formation of a new compartment between these two layers, the periplasmic space (Figure 6). Unlike Gram-positives, whose thick cell wall and external structure is composed largely of peptidoglycan, in the Gram-negative bacteria the cell wall comprises only 10–20% peptidoglycan (Luker et al., 1995). The peptidoglycan structure of Gram-negative bacteria is slightly different than that of Gram-positive bacteria, in particular the free amino group of an unusual diamino acid (often diaminopimelic acid (DAP))

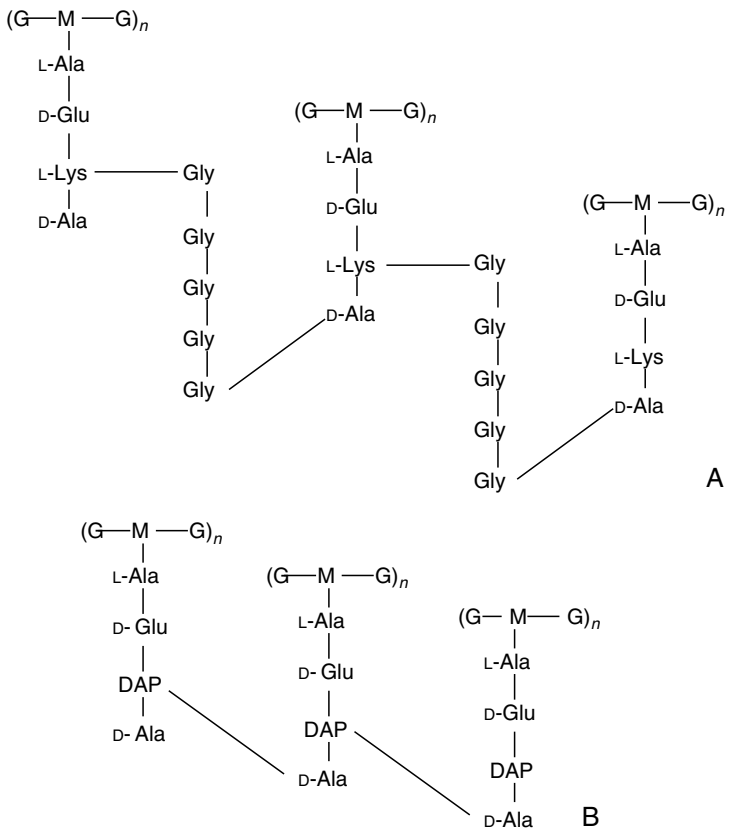


FIGURE 5. (A) Incorporation of peptidoglycan into the *Staphylococcus aureus* cell wall. Pentaglycine chains link individual peptidoglycan subunits together. (B) In *Escherichia coli*, peptidoglycan subunits are linked directly by diaminoacids. This form of linkage is common in Gram-negative and Gram-positive bacteria.

is linked directly to the carboxyl group of D-alanine. The resulting wall, within the periplasmic space, is only about 10 nm thick having only a single layer of peptidoglycan and a lower degree of cross-linking (Table 1), as peptidoglycan subunits in *Escherichia coli* do not have the characteristic pentaglycine bridges observed in the Gram-positive *S. aureus*. Instead, peptidoglycan chains are linked directly from the diamino acid to D-alanine (Figure 5b).

The thin wall of Gram-negative bacteria is highly permeable to hydrophilic molecules. The peptidoglycan layer in Gram-negative bacteria is linked to the outer membrane via a lipoprotein anchor known as lipoprotein. The C-terminal region of lipoprotein binds to the terminal D-alanine of the tetrapeptide, while the hydrophobic N-terminal region, containing the lipid modification, buries itself in the outer membrane, creating a strong linkage between the wall and the outer membrane (Figure 6).

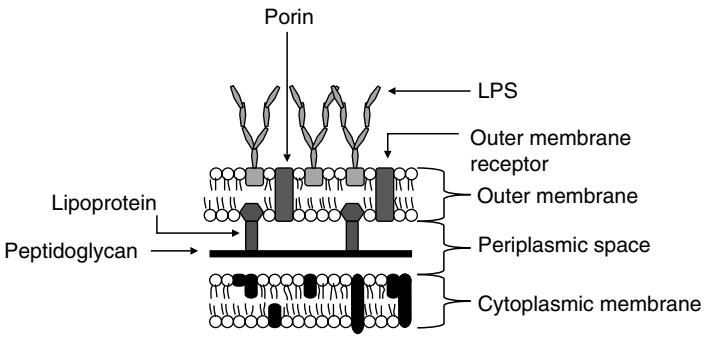


FIGURE 6. Gram-negative cell wall.

In Gram-negative bacteria, LPS predominates in the outer leaflet of the outer membrane, while the inner leaflet of the outer membrane is composed of typical phospholipids. The outer membrane often confers resistance to hydrophobic compounds including detergents, such as bile salts, which are an antibacterial compound encountered by enteric Gram-negative bacteria in the intestinal tract (Thanassi et al., 1997). Resistance to bile salts is important for the adaptation and survival of bacteria in the gut, and pathogenic bacteria (such as *Salmonella* sp.) and commensal bacteria (such as *E. coli*) can utilize a number of mechanisms to survive this antibacterial challenge (Thanassi et al., 1997). Bile can also act as a signal that alters virulence factor production (Gunn, 2000).

LPS, also known as endotoxin, has been shown to be the main component responsible for the toxicity of Gram-negative infections. LPS has two main

TABLE 1. Comparison of Gram-positive vs. Gram-negative bacteria cell walls.

Gram-positive	Gram-negative
Not a barrier to low–molecular weight molecules, including nutrients, antibiotics, dyes, detergents	Outer membrane and porins protect cytoplasmic membrane from these agents; some bacteria are antibiotic resistant because of this
Not a barrier to large, hydrophobic, or nonpolar compounds	
Sensitive to lysozyme and other cell wall lytic enzymes because peptidoglycan is exposed; may have modified peptidoglycan to resist lysozyme	Generally not affected by lysozyme unless outer membrane is breached
Antibody and complement do not kill because attack complex of complement is deposited on the exterior of the cell away from the cytoplasmic membrane	Complement complex can form on outer membrane and have access to cytoplasmic membrane
	Serum resistance can occur by long O antigen LPS, specific proteins, LPS modification
Proteins can be released directly	Have specialized secretion systems for proteins to get through two membranes

components, lipid A and O antigen, that are linked via a conserved carbohydrate core composed of ketodeoxyoctonate, heptoses, and hexoses (Figure 7). LPS inner core sugars can be substituted with charged groups such as phosphates, pyrophosphates, and diphosphoethanolamines (Raetz and Whitfield, 2002). These charged residues can form inter- and intramolecular bonds with divalent cations (calcium and magnesium), effectively stabilizing the LPS molecules (Raetz and Whitfield, 2002).

Lipid A, an unusual glycolipid, comprises fatty acid chains, generally 14 carbons long, which are attached to a disaccharide backbone of β-1,6-linked glucosamines containing multiple phosphate groups. These sugar groups link to the LPS core and are found on the surface of the outer membrane, while the fatty acid chains anchor the LPS molecule into the outer membrane. Lipid A structure can vary slightly between different Gram-negative bacteria and this variation has been suggested to aid in avoidance of recognition by the host immune system (Pulendran et al., 2001). Components of the innate immune system, such as Toll-like receptors (TLRs; see below), e.g., TLR4, can recognize lipid A and mediate subsequent signaling leading to the induction of an inflammatory response (Persing et al., 2002). In many bacteria the genes required for the synthesis of lipid A appear to be essential. This makes these enzymes involved in lipid A synthesis rationale targets for antimicrobial drug design (Gronow and Brade, 2001).

Unlike lipid A, O antigen is highly variable, and is composed of a long chain of carbohydrates up to 40 units in length that vary in composition and order even between members of the same species. In fact, the variability of O antigen is what is used to differentiate strains and subtypes by serotyping using antibodies generated in experimental animals. Because LPS is the immunodominant antigen, each different O antigen can elicit specific antibodies. These antibodies that recognize unique regions on a single form of O antigen can be used to serotype individual strains for O antigen type (Raetz and Whitfield, 2002). Because antibodies are highly specific against individual O antigens, strains of the same bacteria expressing different serotypes may not be eliminated by the immune response and can cause new disease outbreaks. For example, the most recent pandemic of cholera was caused by *Vibrio cholerae* O139, which has an LPS O antigen distinct from that of earlier

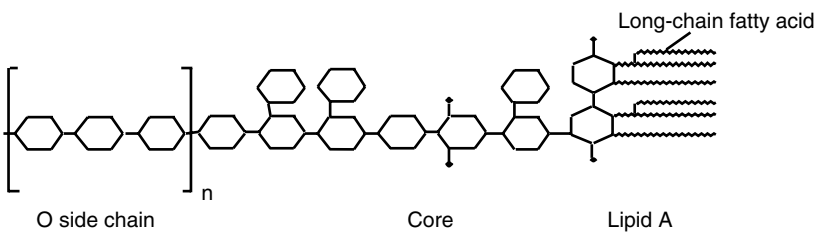


FIGURE 7. Lipopolysaccharide.

outbreaks. It has been suggested that *V. cholerae* O139 Bengal strain originated from a *V. cholerae* O1 strain by homologous recombination-mediated replacement of the genes of the O antigen region (Stroeher and Manning, 1997).

Mutations affecting the O antigen are generally not lethal and are responsible for the phenotype often described as "rough." "Smooth" strains, on the other hand, are those that make an LPS with complete O antigen, which gives colonies a glossy appearance. "Rough" strains do not make a complete LPS, and thus lack the glossy appearance of a "smooth" strain when grown on agar media. One characteristic of "smooth" versus "rough" bacteria relates to their susceptibility to normal human serum. When LPS is complete, the bacteria are resistant to serum, while "rough" strains are serum-sensitive. Indeed, LPS is a central virulence determinant for many bacterial pathogens. Strains of pathogenic bacteria that lack a complete LPS are often attenuated for virulence in animals (West et al., 2003).

The length of O antigen polymer is regulated and important for survival. In the case of gastrointestinal (GI) tract pathogens such as *S. typhimurium*, longer, bulkier polysaccharide chains have been shown to the positively correlate to the protection of this bacterium from phagocytosis, complement-mediated immunity, and confer resistance to bile salts, all of which are encountered during the natural course of infection (Bastin et al., 1993; Murray et al., 2003; Ohno et al., 1995).

Other bacterial pathogens, such as those in the genera *Haemophilus* and *Neisseria* can substitute an oligosaccharide in place of the polysaccharide repeat unit that comprises their O antigen. This short-core oligosaccharide in lipooligosaccharide (LOS) has the same properties as LPS and induces the same endotoxin-like responses and contributes to serum resistance. In severe infections with *Neisseria* spp., LOS is shed into the bloodstream, and can induce disseminated intravascular coagulation (DIC), which is often fatal (Preston et al., 1996; Roth et al., 1992; Uronen-Hansson et al., 2004).

The initial steps of LPS and LOS synthesis occur on the inner leaflet of the inner membrane, where lipid A is synthesized by the addition of fatty acid chains and ketodeoxyoctonate to glucosamine disaccharide. Lipid A embeds in the membrane leaflet and serves as a primer for LPS core synthesis. A variety of specific enzymes add each sugar to the growing core. Once the core is completed, O antigen subunits are transferred to the free end of the core from an undecaprenol-phosphate (und-P) carrier lipid by a transferase. There are two major pathways for the export of O antigen: Wxy-dependent (Figure 8A) and ABC-transporter-dependent (Figure 8B). In both systems, the first step involves the formation of an und-PP-sugar by the transfer of a sugar-1-phosphate to und-P by a protein referred to as WecA. The components of the Wzy-dependent pathway include the Wzy (O antigen polymerase), Wzx (O antigen transporter), and Wzz (O antigen chain-length regulator). After the O unit is assembled, the individual und-PP-linked O units are exposed to the site of polymerization at the periplasmic face of the cytoplasmic membrane.

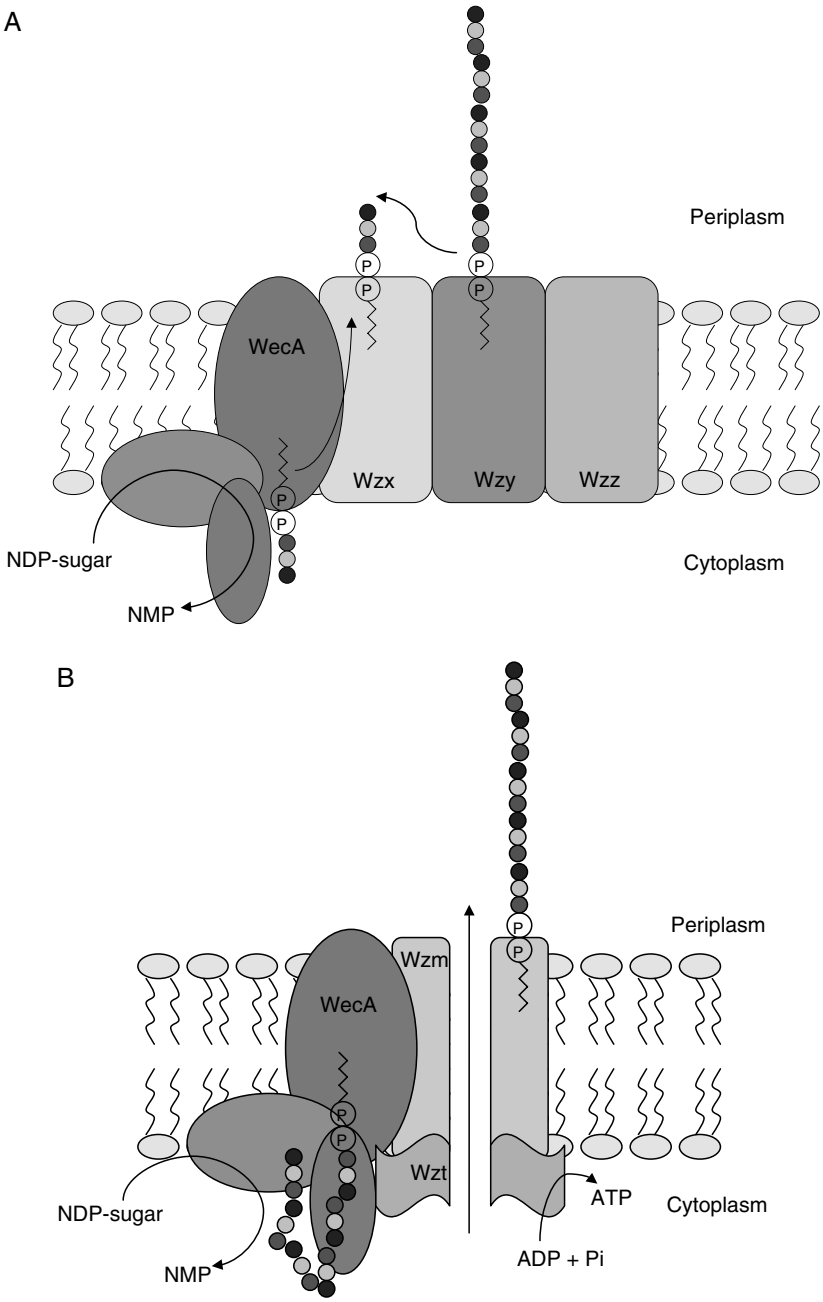


FIGURE 8. (A) Wzy-dependent O antigen pathway. (B) ABC-transporter-dependent O antigen pathway. (After Raetz and Whitfield, 2002.)

Synthesis by the ABC-transporter-dependent pathway is also initiated by WecA. This system includes a Wzm, a membrane-spanning protein, and Wzt, an ATP-binding protein. Following polymerization the O antigen must be exported, but this system does not require a specific polymerase of transporter. The completed LPS molecules are then transferred from the inner membrane to the outer leaflet of the outer membrane though the exact mechanism of this transfer remains unknown (Raetz and Whitfield, 2002).

5. Notable Exceptions

5.1. Acid-fast Bacteria

Mycobacterium tuberculosis, the causative agent of tuberculosis, is the most common cause of death throughout the world due to a single infectious agent. The cell wall of Mycobacteria is neither Gram-positive nor Gram-negative, although it includes elements of both. This unique cell wall comprises three layers: an outer layer of mycolic acid; a middle layer containing the unique sugars arabinogalactan (AG), a polymer of D-galactofuranosyl and D-arabinofuranosyl subunits and lipoarabinomannan (LAM); and an inner layer of peptidoglycan (Figure 9). Mycolic acid, with its branching fatty acids approximately 70–90 carbons in length, forms a lipid layer over the cell wall that is similar to the structure of LPS layers in Gram-negative bacteria. This lipid layer makes up over 10% of the total weight of the Mycobacteria. The inner-facing region of mycolic acids are esterified to AG, which is bound to muramic acid in the peptidoglycan by a linker disaccharide phosphate. AG is responsible for holding the outer and inner layers of the mycobacterial cell wall together, and is necessary for viability (Brennan and Nikaido, 1995) (Figure 9). The waxy layer formed by mycolic acid on the exterior protects the bacteria from killing by phagocytes and is a virulence factor. Because of this layer, common dyes

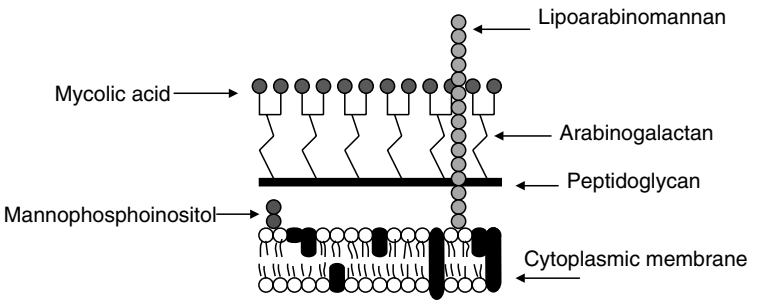


FIGURE 9. The cell wall of Mycobacteria.

used in the Gram stain do not penetrate Mycobacteria. Instead, they are known as acid-fast, for the staining test by which they can be detected. In this test, bacteria are boiled in an acid–alcohol solution with the red dye, fuchsin. Mycobacteria are one of the few that can take up and retain this stain, due to the waxy layer of mycolic acid that prevents the dye from leaching out. Following the acid-fast stain, Mycobacteria appear red, whereas bacteria lacking this layer appear unstained.

The cell wall of Mycobacteria is mitogenic and pyrogenic. In fact, the cell wall is so highly inflammatory that it is used as the main component of Freund's adjuvant. The complex cell wall structure takes a long time to synthesize. It has been suggested that the protracted period required for cell wall synthesis in Mycobacterium relates to the longer doubling time of these organisms. For example *M. tuberculosis* divides once every 18–24 h even under optimal conditions and other Mycobacteria divide every 1–2 h. Despite this slow doubling time, chronic infection with *M. tuberculosis* persists because the bacteria cannot be destroyed by phagocytes and are resistant to most antibiotics. Phagocytes recruited to the site of infection by *M. tuberculosis* are killed, forming a wall of dead phagocytes and necrotic tissue around the site of infection called a granuloma or "tubercule," effectively protecting the bacteria from any further immune response, as well as antibiotics, making late-stage infections difficult to treat (Stewart et al., 2003).

5.2. Mycoplasmas

Unlike Gram-negative and Gram-positive bacteria, Mycoplasmas, such as *Mycoplasma pneumoniae*, the causative agent of primary atypical pneumonia ("walking pneumonia"), have no cell walls. However, they do possess an interesting highly differentiated terminal structure that is thought to be required for cell division, gliding motility, and cytadherence (Krause, 1998). Their cell membranes are more durable than the inner membrane of either Gram-negative or Gram-positive bacteria due to the high concentrations of sterols; yet they are extremely sensitive to osmotic changes. This sensitivity is likely why the shape of mycoplasmas is quite variable. Also, because these bacteria lack typical peptidoglycan-containing cell walls, they are resistant to cell wall–active antibiotics (Hammerschlag, 2001).

6. Cytoplasmic Membrane Components

The cytoplasmic membrane is common to all bacteria. These membranes are composed of phospholipid bilayers and, with the exception of Mycoplasma, these membranes normally do not contain sterols. The major role for this membrane is as a selective osmotic or permeability barrier to regulate the passage of molecules into, and out of, the cells. Similar to eukaryotic membranes, all the known inner membrane proteins have helical transmembrane

segments (Buchanan, 2001). Proteins within the cytoplasmic membrane are involved in a number of critical functions including the transport of nutrients, electron transport, synthesis of peptidoglycan and LPS, machinery for protein assembly and secretion, and regulatory proteins.

Both Gram-positive and Gram-negative bacteria can regulate gene expression to adapt to a variety of environmental conditions encountered in vivo in the infected host and in vitro through a signal transduction system. In the system generally referred to as a two-component regulatory system, a sensor is embedded in the cytoplasmic membrane and then signals to the other component, the response regulator, to bring about a metabolic change. These regulatory systems thus sense the external environmental and activate or repress transcription of a specific set of target genes. In a general setting, changes in environmental conditions first induce the autophosphorylating activity of a transmembrane histidine protein kinase (HPK). The phosphate group from the phosphorylated form of the HPK is then transferred to a specific aspartyl group within the conserved domain of a second protein component. This newly phosphorylated protein serves as a response regulator and can activate the transcription of a specific set of target genes. The genes regulated by two-component systems are diverse, ranging from those for altering the cell surface composition to those required for virulence gene expression (see Chapter 13).

7. Externally Exposed Structures

Structures on the outer surface of the bacterial cell can interact directly with the environment. Because of their exposure during infection, these structures can also serve as targets for the immune system. To evade the host immune response, these structures may be variably expressed or show alterations in antigenic characteristics.

A capsule layer can surround the outer surface of some bacteria. This structure can function as a protective coat against dehydration or toxic compounds. Capsules in some cases mediate adherence, while in other cases they appear to mask the bacterial cell surface and decrease adherence. Additional roles for these structures include protecting the bacteria from engulfment by phagocytic cells (Roberts, 1996). In many cases the capsule is composed of polysaccharide that may be loosely attached to the cell surface. However, the capsule of *Bacillus anthracis*, the causative agent of anthrax, has a polypeptide capsule of polyglutamic acid (Ezzell and Welkos, 1999). Many capsular polysaccharides are weakly immunogenic. In the case of *S. pneumoniae*, the leading cause of bacterial pneumonia, the polysaccharide is a major virulence factor and serves as the basis of serotyping (Casal and Tarrago, 2003). In this and other cases, polysaccharides have served as antigens for vaccine development (Bogaert et al., 2004; Ezzell and Welkos, 1999; Pier, 2003; Zimmer and Stephens, 2004). Due to their general lack of immunogenicity, vaccines against polysaccharides require conjugation to peptide or

protein carriers to potentiate their antigenicity (Lesinski and Westernik, 2001; Weintraub, 2003) (see Chapter 5).

The S-layer is a crystalline structure that can also be found external to the peptidoglycan layer in Gram-positive bacteria and the outer membrane in Gram-negative bacteria (Sara and Sleytr, 2000). This layer is usually composed of a single self-assembling protein (or glycoprotein) and forms a two-dimensional lattice-like structure. These S-proteins can constitute between 5% and 10% of the total protein content of the bacteria. In addition, S-proteins from both Gram-positive and Gram-negative bacteria can interact with components of the cell surface. The presence of this layer on the outer surface suggests that it has a role in protection from environmental stresses and adherence. Likely due to these properties, the S-layer has been shown to contribute to virulence by promoting adherence and resistance to bactericidal activity of complement (Sara and Sleytr, 2000). In addition, because of this surface accessibility and high level of expression, the S-layer of Lactobacillus, for example, has been proposed as model system to display foreign antigen epitopes for vaccine development (Avall-Jaaskelainen et al., 2002).

7.1. Flagella

Flagella are thin, threadlike organelles associated with motility that are found on the surface of both Gram-positive and Gram-negative bacteria and are generally too thin (~0.2 μm) to be detected by light microscopy. These thin structures comprise primarily the protein flagellin, which forms a long filamentous fiber that weaves around itself to form a left-handed helix (Normile, 2001). The helix is embedded in a basal body, and is anchored to the cell membrane, forming the structure of a single flagellum. The localization of the flagella on the bacteria can be characteristic of a particular genus (Figure 10). In general, when the bacteria are subjected to an attractant, the rings around the basal body rotate relative to each other making the flagella move. If the flagella rotate counterclockwise, they will bunch together and propel the bacteria forward in a straight line or run toward an attractant. If rotated clockwise, the flagella will spread and send the bacteria tumbling through the media away from a repellent.

Flagella have been shown to have important roles in virulence. In the case of *Helicobacter pylori*, for example, the flagella have been shown to be critical for the ability of the bacteria to migrate through the mucous layer of the stomach to interact with epithelial cells as well as be protected from the low pH of the stomach. Aflagellated mutants of *H. pylori* have been shown to be less virulent in animal models of infection (Ottemann and Miller, 1997). Similar results have been obtained with nonflagellated mutants of *Pseudomonas aeruginosa*. In contrast to the wild-type strain, an isogenic *P. aeruginosa* strain mutated in the major flagellin subunit did not cause mortality in a neonatal model of infection (Feldman et al., 1998). Subsequent studies have noted that conserved site within the flagellin protein from both Gram-positive and Gram-negative bacteria

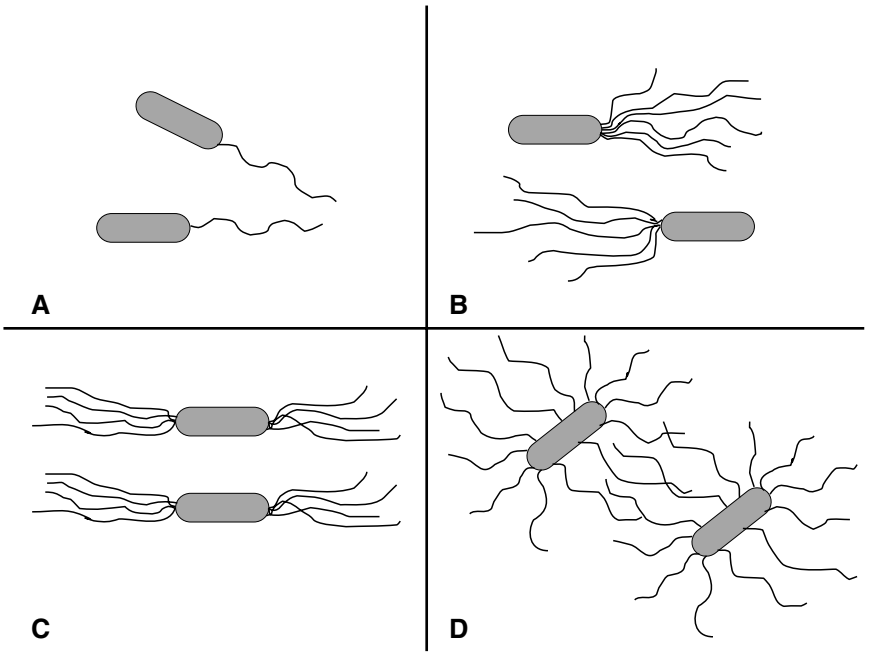


FIGURE 10. Flagellar placement on the surface of a bacterium varies substantially across genera. The placement may be (A) singular or monotrichaete; (B) concentrated at a single point or lophotrichaete; (C) concentrated at the polar ends of the bacterium or amphitrichaete; or (D) dispersed equally over the entire bacterial surface, as in the case of a petritrichaete.

activates the innate immune system through its recognition by TLR5 (Smith et al., 2003) (see below).

7.2. Pili

Pili are hairlike structures built with protein subunits called pilins. These usually extend 1–2 μm from the bacterial cell surface and can have diameters of 2–8 nm. Type I and type IV pili are the best characterized of these structures and are found ubiquitously on Gram-negative pathogens (Craig et al., 2004; Hung and Hultgren, 1998) (see Chapter 7). In addition, there are less abundant structures like the F (fertility) and other conjugal pili (Kalkum et al., 2002). These latter pili are long, hollow tubules, primarily involved in conjugation, where proteins on the pili tips bind to a recipient cell and bring it into close contact with the donor cell for DNA exchange.

Type I pili of uropathogenic *E. coli* (Figure 11) are encoded by the *fim* operon. FimA is the major pilin subunit and FimH is the adhesin that recognizes β-D-mannosides. FimC and FimD are the periplasmic chaperone and

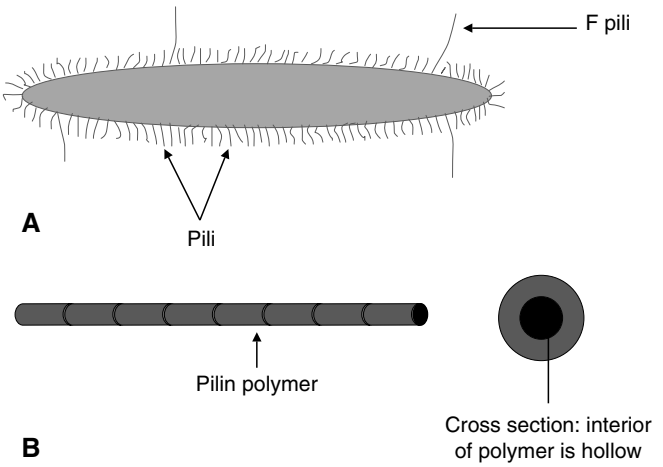


FIGURE 11. (A) Pili (or fimbrae) are short, bristle-like fibers found abundantly on the surface of bacteria often involved in adherence. F pili are much longer than fimbrae and are involved in conjugation. (B) Pili are long, hollow tubules of polymerized pilin.

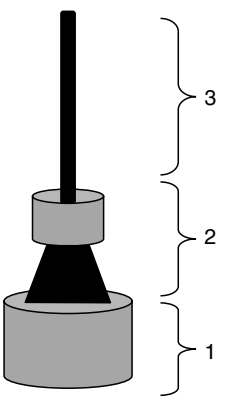
outer membrane usher, respectively—components of the specific assembly machinery. Proteins encoded by the *fim* operon are synthesized as precursor with canonical N-terminal signal peptides that directs them through the cytoplasmic membrane to the periplasmic space using the general secretory (Sec) pathway (Fernandez and Berenguer, 2000). Both phase and antigenic mechanisms of variation have been noted to be involved in the regulation in a number of type I pili that allow bacteria to target different cell types and specific localities as well as adjust to the host immune response.

Type IV pili have been shown to be important for adherence to host tissues in a number of important pathogens, including *V. cholerae* and *P. aeruginosa*, various *E. coli* strains, and some *Neisseria* spp. (Craig et al., 2004; Mattick, 2002). The pili are synthesized as precursors with short basic N-terminal signal peptides. The pre-pilin are cleaved and also *N*-methylated at the first amino acid of the mature pilin. All these pili have homology in the N-terminal and C-terminal regions and a pair of cysteines that form a disulfide bridge in the mature protein and use similar secretion and assembly components. Secretion and assembly involves at least 12 proteins, which are related to the type II secretion pathway for export. Type IV pili are also involved in nonflagella movement, which is referred to as "twitching motility." This type of movement is caused by the reversible polymerization and depolymerization of pilus subunits. In *P. aeruginosa*, "twitching motility" has also been shown to be important for biofilm formation and pathogenesis in some models of infection (Mattick, 2002).

7.3. Type III Secretion Apparatus

Type III secretion is found only in Gram-negative pathogens and is highly adapted to delivering virulence factors from bacteria directly into the eukaryotic cell (Thomas and Finlay, 2003). The apparatus may be composed of more than 20 genes. It is a complex protein secretion system, requiring a multitude of chaperones for many of the secreted proteins as well as direct contact with the epithelial host cell to initiate activation of the system (Thomas and Finlay, 2003). The origins of the type III secretion system are not fully known, but the similarity of the basal structure genes to basal flagellar genes suggests that it may have originally evolved as a flagellar export system varied to transport proteins other than flagellin to the cell exterior (He, 1998). Because the type III secretion machinery genes in plant pathogens share a higher homology with their basal flagellar genes than animal pathogens, it is tempting to speculate that the original prototype of the type III secretion apparatus may have developed in plant pathogens and was transferred to animal pathogens by horizontal gene transfer (He, 1998).

In *S. typhimurium*, the external needle of the type III secretion apparatus is composed of three main parts: a long, hollow needle; a transmembrane domain; and a small cylindrical base. The tubule extends from the bacterial surface to a length of approximately 120 nm, and comprises mainly InvG, a member of the secretin family (Collazo and Galan, 1997; Galan, 1997; Pratt et al., 1996). The cylindrical base found at the bottom of this needle is composed of two lipoproteins, PrgH and PrgK, which firmly anchor the needle to the bacterial membrane (Collazo and Galan, 1997; Galan, 1997; Pratt et al., 1996) (Figure 12). The mechanism and stimulus by which the secretion system is activated varies by species, but always requires contact with a host



1. Cylindrical base
2. Transmembrane domain
3. Needle

FIGURE 12. Type III secretion apparatus.

cell or a similar signal. In *Yersinia pestis*, which is the causative agent of bubonic plague, the apparatus sits in a closed position until the bacteria comes into contact with a phagocyte. Upon contact, the needle springs outward, much like the needle of a lancet, and transports effector proteins and their chaperones across the membrane into the host cell cytosol (Collazo and Galan, 1997; Galan, 1997; Pratt et al., 1996) (see Chapter 9).

7.4. Porins

In Gram-negative bacteria, outer membrane proteins have unusual β-barrel structures (Nikaido, 2003). These proteins are stable to denaturation, solubilization in detergent, and protease digestion. Porins are some of the most abundant outer membrane proteins and allow the transport of small hydrophilic molecules through the outer membrane and aid the bacteria in adapting to a number of environmentally stressful conditions such as phosphate limitation or low osmolarity. Porins are protein trimers that form a pore in the membrane (typically only 1–2 nm in diameter) (Figure 13), and

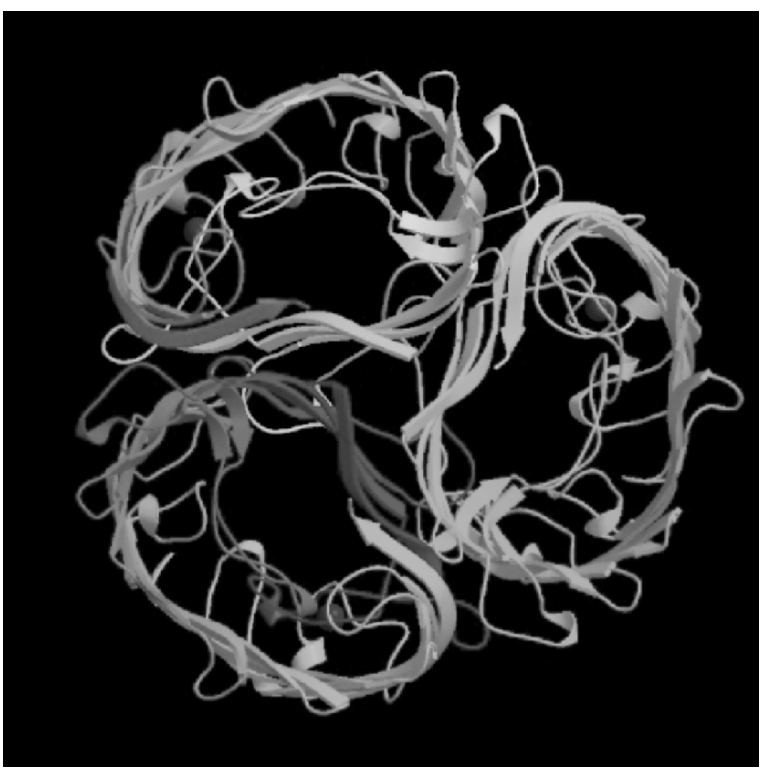


FIGURE 13. 3D structure of bacterial porin 1AOS from the protein data bank.

bind noncovalently to the peptidoglycan wall, remaining attached when the outer membrane has been dissolved by detergent. Each bacterial cell may have many types of porins that may be inducible by different environmental conditions. In *E. coli*, two major porins are expressed in the outer membrane, OmpC and OmpF, which allow the transport of molecules 500 Da and 600 Da, respectively (Massari et al., 2003; Pratt et al., 1996). Under conditions of high osmolarity, OmpC is present, allowing only molecules of 500 Da and smaller to pass through the membrane pore. However, under conditions of low osmolarity, OmpF is more prominent than OmpC to allow the transport of larger molecules. In the case of *Neisseria*, the outer membrane porins are the most abundant outer membrane proteins (Del'vig and Semenov, 1997; Koomey, 2001; Massari et al., 2003). In addition to their role as channels, these porins have been shown to be important for bacterial interactions with host cells including attachment and internalization. These proteins also appear to play a role in serum resistance, induction of apoptosis and B-cell activation (Koomey, 2001).

7.5. Outer Membrane Transporters

In the outer membrane of Gram-negative bacteria, there are proteins that allow the transport of molecules too large or too scarce to accumulate via diffusion through the nonspecific and substrate-specific protein porins. These specialized, ligand-specific, active transport proteins move compounds against a concentration gradient across the outer membrane. In each case the energy for this transport is derived from the cytoplasmic membrane proton motive force via interactions with the inner membrane protein TonB, and is associated with ExbB and ExbD in the inner membrane. These OM transporters can also be exploited as receptors by bacteriophages and bacteriocins (Postle and Kadner, 2003).

7.6. Efflux Pumps

Efflux pumps are one major mechanism by which bacteria have become resistant to chemicals, and can be either specific for a given class of antimicrobial agents or able to remove a broad variety of unrelated compounds. Efflux pumps are classified into five or six major families, owing to the specific mechanism by which they function (Borges-Walmsley et al., 2003; Li and Nikaido, 2004). The resistance-nodulation-division (RND) family is the most important for clinically relevant Gram-negative bacteria, but this family has now been found in all kingdoms. In Gram-negative bacteria the RND system has three components: a transporter located in the inner membrane that functions with an outer membrane protein and a periplasmic accessory protein; the three components are often encoded in the same gene cluster. The proteins of this tripartite system both traverse the outer membrane to facilitate passage of substrate from the cytoplasm

into the external media and allow the efflux of, and resistance to, a variety of clinically important antibiotics and biocides. These transporters are predicted to utilize the proton motive force to promote export of compounds from the cell (Poole, 2001). In Gram-positive bacteria these systems depend on the energy of ATP hydrolysis or transmembrane electrochemical gradients to actively transport compounds out of the cell. Because Gram-positives do not have an outer membrane, these membrane transporters are the only means to actively pump antibiotics out of the cell (Markham and Neyfakh, 2001).

8. Cell Wall Antibiotics

Many antibiotics have been developed to target the bacterial cell wall, since it has no homologous structure in mammalian cells. Thus, drugs disrupting only this structure eventually lead to death of the infecting bacteria. However, release of cell wall and cell contents can have toxic effects due to their interaction with the immune system. This should be taken into consideration when any antibiotic is prescribed.

Antibiotics targeting bacteria fall generally into one of two categories: bactericidal and bacteriostatic. Bactericidal drugs require bacterial growth to kill bacteria, and drugs targeting the cell wall often fall into this category, requiring active synthesis and cell wall growth to function. Bacteriostatic drugs inhibit further bacterial growth but do not kill the infecting organism, relying instead on the immune system of the host to destroy the nonreplicating bacteria. Listed below are some examples of cell wall–specific antibiotics that are commonly used to treat bacterial infections and their mechanisms of action (Figure 4B).

8.1. Antibiotics Affecting Early Steps in Peptidoglycan Synthesis

Fosfomycin is an analog of phosphenolpyruvate. It binds covalently to muramic acid synthesizing enzymes to prevent synthesis of the muramic acid precursor. Cycloserine inhibits the synthesis of the D-ala-D-ala dipeptide of the terminal residues of the pentapeptide chain (Noda et al., 2004).

Bacitracin prevents the dephosphorylation and recycling of the lipid carrier after *N*-acetyl glucosamine-*N*-acetyl muramic acid donation. This drug may also prevent phosphorylation in similar lipid carriers in mammalian cells. Thus, it is fairly toxic to humans if taken internally, and is commonly used only in topical applications to wounds and surface infections.

Vancomycin is only effective against Gram-positive bacteria, as it is prevented from reaching its site of action in Gram-negative bacteria due to its large size. Vancomycin is a glycopeptide antibiotic that functions by binding to the D-alanyl-D-alanine end of the peptidoglycan precursor and blocks

transglycosylation and transpeptidation, thus preventing incorporation of the subunit into the existing wall structure. It has been used especially to target infections by methicillin-resistant *S. aureus*, as it is one of the few antibiotics that this species remains sensitive to. However, vancomycin-resistant *S. aureus* strains have now been isolated; the resistance was likely due to the interspecies transfer of a genetic element from *Enterococcus faecalis* (Weigel et al., 2003). As *S. aureus* is one of the main causes of potentially lethal infections, especially in hospital settings, this mechanism presents a major concern to the health system for the development of widespread antibiotic resistance.

8.2. β-Lactam Antibiotics

β-lactam antibiotics are perhaps the oldest and best known in the world today; penicillin was discovered by Sir Alexander Fleming in 1929. The β-lactam ring of this class of antibiotics mimics the D-alanyl-D-alanine bond found in peptidoglycan precursors, and is bound by penicillin-binding proteins prior to being integrated into the cell wall as if they were a peptidoglycan subunit. However, because penicillin binds to the enzymes that carry out the cross-linking, new peptidoglycan subunits cannot be added in the vicinity of the antibiotic. As the bacterium continues to grow, these patches are never sealed, and the bacteria lyse due to osmotic pressure from the environment. The β-lactam family is large, with each drug having different side chains attached to the conserved β-lactam ring (Figure 14). The nature of the side chain gives these drugs different properties including stability, permeability, and resistance to β-lactamases.

8.3. Isoniazid

Isoniazid is primarily used to treat mycobacterial infections, such as tuberculosis, where it blocks the function of InhA, an NADH-dependent enoyl-ACP reductase, which has a preference for long-chain substrates. Inactivation of InhA prevents mycolic acid synthesis, leads to the accumulation of 26-carbon fatty acids and morphological changes and eventually cell lysis (Heath et al., 2001). Isoniazid is bacteriostatic against resting bacilli and bactericidal against multiplying organisms. Aside from resistance that was noted soon after its introduction, there are adverse side effects to isoniazid including liver toxicity.

9. Antibiotic Resistance

Resistance to cell wall targeting antibiotics can occur through many mechanisms, the most common of which use enzymes known as β-lactamases, which destroy the β-lactam rings of antibiotics before the molecules can be

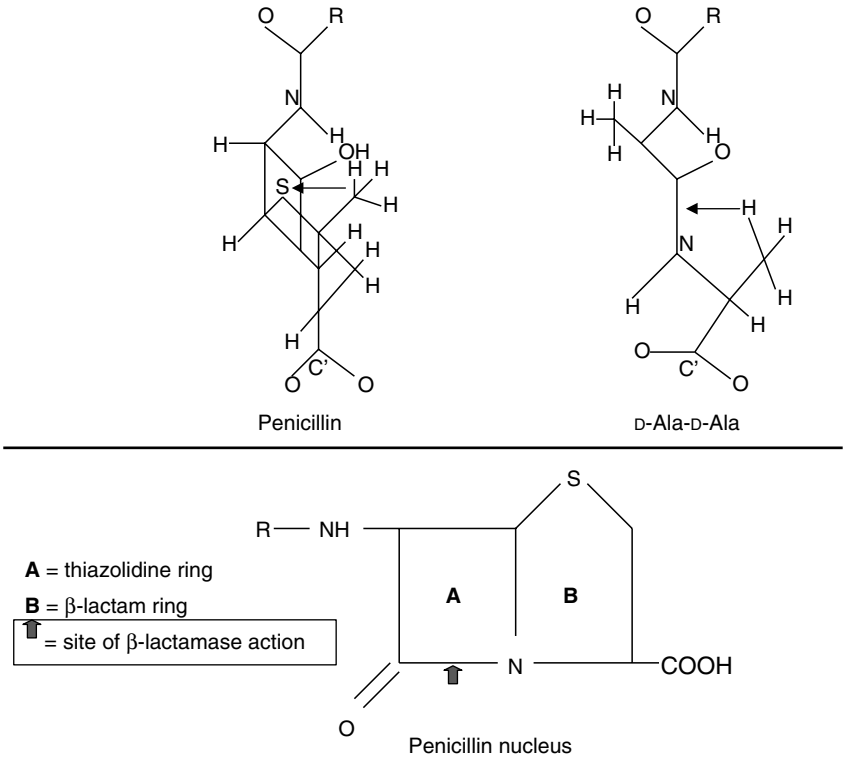


FIGURE 14. β-lactam ring structure.

inserted into the growing wall. However, some β-lactam antibiotics, like methicillin, are less susceptible to β-lactamase activity.

Because β-lactam antibiotics target the same structure of the cell wall, resistance is often cross-reactive; resistance to one member of the β-lactam family often confers resistance to other members of the antibiotic class (Amyes, 2003; Shah et al., 2004). Similarly, resistance to triclosan, an agent frequently found in antibacterial compounds, inhibits fatty acid biosynthesis, and can result in cross-resistance to isoniazid. Emergence of resistance to isoniazid is cause for some alarm as this is one of the few antibiotics for treating *M. tuberculosis* infections.

Resistance to other antibiotics can also occur through the acquisition of molecular pumps, which selectively pump antibiotics out of the bacterium and back into the environment, through alterations to the structure of the target, or through modification of the antibiotic, making it ineffective. For example, tetracycline resistance can be caused by activation of an efflux pump; vancomycin resistance can be due to alterations in the structure of the peptidoglycan; and chloramphenicol resistance can be caused by acetylation

of the antibiotic leading to inhibition. To further complicate the evolution of resistance, bacteria need not be subjected to antibiotic treatment to acquire resistance. Plasmids can carry genes encoding antibiotic resistance and can be shared between different strains of bacteria through transformation, transduction, and conjugation (see Chapter 2).

10. Innate Immune Response to Cell Wall Components

The innate immune system is essential for early detection of potential pathogens. Toll-like receptors (TLRs) are a family of receptors that recognize conserved motifs that are unique to microorganisms, called pathogen-associated molecular patterns. Related to the *Drosophila* Toll receptors (Koopp and Medzhitov, 2003), TLRs are found on immune cells such as macrophages and dendritic cells as well as epithelial and endothelial cells (Beutler, 2004; Li et al., 2004; Netea et al., 2004). There are 11 human TLRs so far identified, with different TLRs recognizing different pAMPs (Akira and Takeda, 2004; Werling and Jungi, 2003) (Table 2). For example, the ligand for TLR2 is peptidoglycan and lipoteichoic acid, the ligand for TLR4 is generally LPS, and TLR5 recognizes the flagellin subunit of bacterial flagella (Beutler, 2004; Li et al., 2004; Netea et al., 2004). Once a ligand interacts with the TLRs, a signaling cascade is initiated, causing activation of the transcription nuclear factor-κB (NF-κB), as well as members of the mitogen-activated protein kinases (MAPKs) (Dalpke and Heeg, 2002; Dunne and O'Neill, 2003; Werling and Jungi, 2003). This pathway ultimately results in the transcription of a variety of host defense genes, including interleukin-8 (IL-8), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), and interleukin-1 (IL-1), which are responsible for the initiation of the inflammatory response (Dalpke and Heeg, 2002; Dunne and O'Neill, 2003; Werling and Jungi, 2003). In some cases, it could be suggested that inhibiting this process could benefit the host by reducing the consequences of a damaging immune response. However,

Table 2. Toll-like receptors and their cognate ligands.

TLR1	In conjunction with TLR2
TLR2	Peptidoglycan, lipoproteins, lipoteichoic acid, glycolipids, atypical LPS, lipoara binomannan, etc.
TLR3	Double-stranded RNA
TLR4	LPS, lipotheichoic acid
TLR5	Flagellin
TLR6	In conjunction with TLR2
TLR7	Imidazoquinolines, single-stranded RNA
TLR8	Imidazoquinolines, single-stranded RNA
TLR9	Unmethylated CpG DNA
TLR10	Unknown
TLR11	Unknown

care must be taken in such an approach as intervention may also interfere with the beneficial aspects of the normal host response.

11. Conclusions

Cell walls are crucial for the adaptation and survival of bacteria in a wide range of environmental conditions, including those encountered by pathogenic species during infection. The functions of the cell wall are diverse, ranging from protection from osmotic lysis to the evasion of host immune responses and the expression of structures required for interaction with the host cell. Future research in the synthesis and regulation of bacterial cell walls will be needed to uncover the intricate mechanisms by which these structures are created and maintained, and will undoubtedly lead to the discovery of novel targets for a new generation of antibiotics.

Questions to Consider

1. Compare and contrast the differences and similarities of Gram-positive and Gram-negative bacteria.

Microscopic observation after Gram staining reveals Gram-positive bacteria appear blue while Gram-negative bacteria appear red. Also, refer to Table 1.

2. How do autolysins function in cell wall synthesis?

Autolysins dissolve patches of cell wall and allow new peptidoglycan units to be inserted during bacterial cell growth.

3. What are the roles of lipopolysaccharide (LPS) for Gram-negative bacterium?

The O antigen of LPS confers serotype specificity. Long O antigens are also responsible for serum resistance and resistance to detergents. The lipid A portion of LPS is endotoxic and interacts with Toll-like receptors (TLRs) of the innate immune system. LPS components have also been shown to act as ligands to host tissues.

4. Why are Mycobacteria not stained in a Gram stain?

The mycolic acid layer forms a waxy wall around the bacteria that resists penetration by the Gram stain. Specific staining techniques referred to as acid-fast staining are required to visualize these bacteria microscopically.

5. Why are Mycoplasma generally resistant to cell wall–active antibiotics?

Mycoplasma do not have peptidoglycan in their membranes. These membranes do contain sterols making them durable, but highly sensitive to osmotic changes.

6. What are some of the functions of porins?

Generally considered channels through which molecules can pass into the cell, in some systems porins can also have a role in serum resistance, induction of apoptosis, and B-cell activation. These structures have also been shown to be important for attachment and internalization into host cells.

7. Why do so many antibiotics target the cell wall? What are the problems associated with these antibiotics?

The cell wall is generally an accessible structure and is integral to the bacteria's cellular integrity. Homologous cell wall structures are not found in mammalian cells and, therefore, cell wall targets are specific. However, release of cellular contents by treatment with cell wall–active antibiotics may lead to toxic effects.

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Chapter 7

Mechanisms of Bacterial Adhesion and Consequences of Attachment

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Historical Landmarks

1989 The first structure of a periplasmic chaperone, PapD solved (Holmgren et al. 1989).

1995 Identification of PilC as the adhesin of neisserial type IV pili (Rudel et al., 1995).

1995 Crystal structure of PilE, the major structural monomer of neisserial type IV pili, solved. (Parge et al., 1995).

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1998 Genetic basis of type IV pilus retraction characterized (Wolfgang et al., 1998, 2000).

1999 Crystal structures of the FimC chaperone complexed with the FimH adhesin and the PapD chaperone complexed with the PapK subunit solved (Choudhury et al. 1999; Sauer et al., 1999).

1999 Model proposed for donor strand complementation and donor strand exchange during biogenesis of chaperone/usher pili (Choudhury et al., 1999; Sauer et al., 1999).

2001 Crystal structure of PapG bound to its globoside receptor solved (Dodson et al., 2001).

2003 Crystal structure of chaperone/subunit complex formed during biogenesis of an afimbrial adhesive organelle from *Yersinia pestis*, F1 capsular antigen, solved (Zavialov et al., 2003).

1. Introduction

Binding to surfaces is an important event in the life cycle of virtually all microorganisms. Binding allows bacteria to persist at advantageous locations, where there may be a concentration of nutrients or protective environments. One of the very early events in most infection processes involves binding of the causative microorganism to host receptors at the primary site of infection. This binding, usually mediated by microbially produced adhesins expressed on their extracellular surface, provides bacteria with a base from which to proliferate, colonize, and cause damage to the epithelium. Alternatively, attachment can lead to invasion of the bacteria into host tissues in an effort to evade host immune defenses, which are activated upon bacterial infiltration. Many adhesive structures of bacteria also undergo tight regulation and antigenic variation to subvert these host defenses. This complicated cross-talk at the host–pathogen interface demonstrates the vast significance of attachment and the clever strategies bacteria use to manipulate their host during pathogenesis.

Generally, binding involves a specific interaction between an adhesin and its receptor. It is this specificity that determines tropism of the microorganism to a particular host tissue. Often, bacteria possess multiple adhesins, which allow the pathogen to attach to several different sites in both the host and the environment. The ability to exploit multiple niches provides the pathogen with versatility and options as to how and where it wants to colonize. Moreover, displaying multiple adhesins increases the chances that a pathogen will be able to persist in preferred locations, as well as be able to be transmitted to new sites. In this sense, bacterial attachment is one of the most important qualities that a pathogen must possess.

2. Diversity of Adhesins

Considering the numerous locations a potential pathogen can infect, and the incredible diversity of molecules present on tissue surfaces that could be exploited as receptors; it is not surprising that a multitude of adhesins and adhesive structures are produced by bacteria. Many organisms assemble adhesins at the tip of macromolecular fiber-like appendages, called fimbriae or pili, which reach out to grab distant receptors. Another adhesive strategy is the production of afimbrial adhesins, which initiate more intimate interactions. Many bacteria use common structures present on their surface, such as capsules and flagella, in order to bind to host tissues. However, some of the most widespread adhesins among bacteria are those associated with pili assembled via the chaperone/usher pathway.

2.1. The Chaperone/Usher Pathway of Adhesin Assembly

The chaperone/usher pathway is involved in the construction of more than 30 different structures in pathogenic Gram-negative bacteria (Thanassi et al., 1998a). Pilus subunits, regulatory elements, and accessory factors are typically arranged into operons on the bacterial chromosome (Jones et al., 1996). Two key proteins encoded by chaperone/usher operons are a periplasmic chaperone and an outer membrane usher, which assist in the incorporation of pilus subunits into large macromolecular assemblies protruding from the outer membrane (Sauer et al., 2000). Upon transcription and translation, individual subunits are translocated into the periplasm through the general secretory pathway (Sauer et al., 2000). Immediately after translocation, interaction of the subunits with the periplasmic chaperone assists periplasmic entry, prevents subunits from forming nonproductive interactions by capping interactive surfaces, and assists folding of subunits into their proper conformations (Jones et al., 1996; Sauer et al., 2000). Subunits then traverse across the outer membrane through the usher, where they are assembled into the pilus rods that extend from the bacterial surface (Thanassi et al., 1998a). Much of what is known about the chaperone/usher pathway of pilus assembly comes from studies of two pili of *Escherichia coli*: type 1 pili and P pili.

2.1.1. Type 1 Pili

Type 1 pili are expressed by many members of the bacterial family *Enterobacteriaceae*, including *E. coli* (Soto and Hultgren, 1999). The FimH adhesin, located at the tip of type 1 pili, recognizes mannose residues on host cell receptors. For *E. coli*, FimH-mediated binding to mannosylated receptors is vital for colonization and establishment of infection in the urinary tract (Mulvey et al., 1998), and type 1 pili have also been shown to be important for biofilm formation (Pratt and Kolter, 1998). Type 1 pili assemble into

7 nm-wide helical rods extending up to several micrometers from the outer membrane of *E. coli*, with a thinner tip fibrillum at the distal end of each pilus (Jones et al., 1995; Soto and Hultgren, 1999). The type 1 pilus operon encodes for the major structural subunit (FimA), a periplasmic chaperone (FimC), an outer membrane usher (FimD), two pilus adaptor proteins (FimG and FimF), and an adhesin (FimH) (Figure 1A). FimH is located at the tip of type 1 pili, followed by FimG and FimF, forming the thin tip fibrillum (Jones et al., 1995). Repeating monomers of FimA constitute the majority of the type 1 pilus structure (Jones et al., 1996). Kinetic studies of subunit–usher interactions have shown that, during pilus assembly, the FimH adhesin (in association with the FimC chaperone) has high affinity for the FimD usher, and the other subunits (FimG, FimF, and FimA) associate with the usher at varying, lesser affinities (Saulino et al., 1998). This establishes a kinetic partitioning which ensures that the FimH adhesin is the first subunit of each pilus rod, followed by the FimG and FimF adaptor proteins. Finally, numerous FimA monomers are recruited to form the bulk of the growing fiber at the outer membrane to the type 1 pilus rod (Jones et al., 1996) (Figure 1B).

Construction of ordered pilus rods is strictly regulated by phase variation, which has been shown to be important during urinary tract infections (Gunther et al., 2001). The promoter of the type 1 pilus operon is located on an invertible element (Gunther et al., 2001). The orientation of this element depends upon two recombinases, encoded by *fimB* and *fimE*, located upstream of this promoter region (Schwan et al., 2002) (Figure 1A). FimB facilitates switching from the phase-off to phase-on orientation or from phase-on to phase-off. FimE, on the other hand, generally seems to switch from phase-on to phase-off, although it has been suggested that it might also influence off-to-on switching as well (Schwan et al., 2002). Environmental factors from the surrounding milieu influence the interplay between these recombinases and, consequently, the expression of the type 1 pilus operon (Schwan et al., 2002).

Upon expression, nascent subunit monomers are translocated through the general secretory pathway into the periplasm, where they are bound by the FimC chaperone (Figure 2). Structural and biochemical studies of FimC have shown that this chaperone consists of two immunoglobulin (Ig)-like domains (Pellecchia et al., 1998). Protein domains of the Ig-like family are characterized by 7–9 β-strands. These strands fold into two β-sheets that pack against each other (Bork et al., 1994). The Ig-like domains of FimC each have seven β-strands and are arranged in the shape of a boomerang connected by a short linker region (Choudhury et al., 1999; Pellecchia et al., 1998; Sauer et al., 2000) (Figure 2). Similar studies have demonstrated that the pilus subunits each contain a single Ig-like domain. However, pilus subunits are missing their carboxyl (C)-terminal β-strand (Choudhury et al., 1999; Sauer et al., 1999). This creates a deep groove on the subunit, leading to instability in the absence of the chaperone (Barnhart et al., 2000; Jones et al., 1997; Sauer et al., 2000). The FimC chaperone stabilizes the pilus

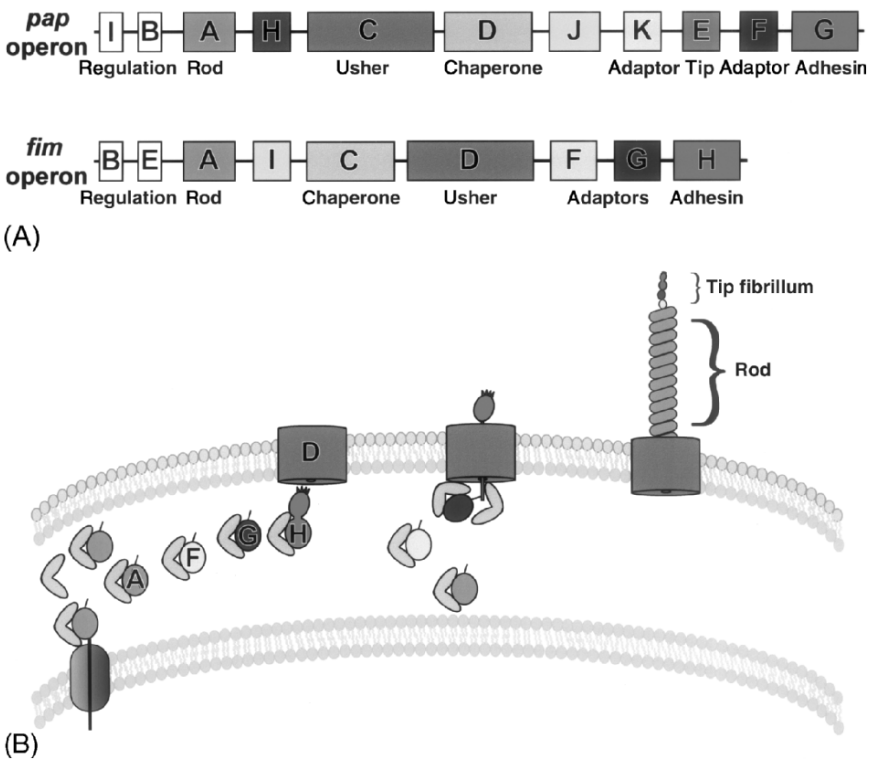

FIGURE 1. Genetic organization and biogenesis of type 1 and P pili. (A) The *pap* and *fim* operons, encoding for P pili and type 1 pili, respectively. The operons are preceded by regulatory elements that affect the pilus expression status. Genes in each operon encode for the major structural subunit of the pilus rods, an outer membrane usher, a periplasmic chaperone, adaptor subunits of the tip fibrillum, and an adhesin. (B) The biogenesis scheme of type 1 pili. The colors of each player in the pathway correspond with the colors of the *fim* operon genes in (A). Subunits are translocated into the periplasm through the general secretory pathway (blue oval). In the periplasm, the FimC chaperone binds to individual subunits, stabilizes them by donor strand complementation, and transports them to the outer membrane usher, FimD. Subunits are translocated across the outer membrane through the usher in an extended conformation. Incoming chaperone/subunit complexes displace the chaperone of the previous subunit as the N-terminal extension of the newly arrived subunit completes the Ig fold of the subunit before it by donor strand exchange. The adhesin FimH is displayed at the tip of the pilus and forms the thin tip fibrillum along with FimG and FimF. Repeating monomers of FimA form the major helical structure of the pilus rod.

subunit by transiently donating its G1β strand to fill this groove in a process termed "donor strand complementation" (Choudhury et al., 1999; Sauer et al., 1999). Donor strand complementation completes the Ig fold of subunits in an atypical manner whereby the G1β strand of the chaperone runs

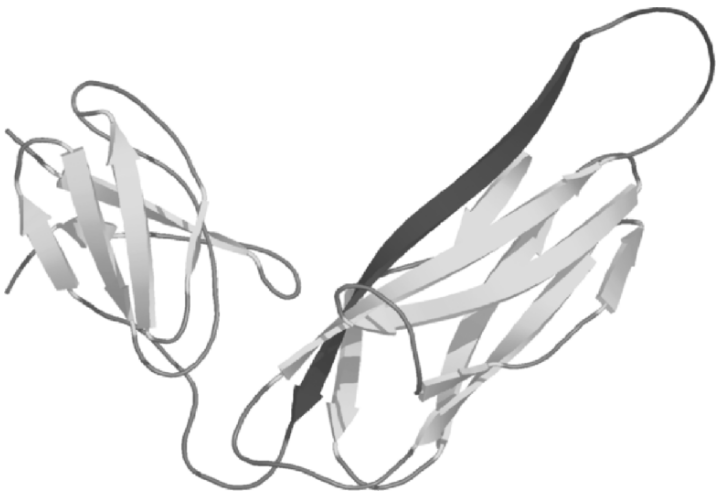

FIGURE 2. Crystal structure of the FimC chaperone. FimC contains two Ig-like domains, which orient themselves in the shape of a boomerang with a short linker region. FimC stabilizes and assists folding of pilus subunits by donating its G1β strand to complete the Ig fold of the subunits. Structural coordinates from PBD ID 1KLF (Hung et al., 2002).

parallel to the C-terminal β-strand of the subunit. Chaperone/subunit complexes are then targeted to the usher at the outer membrane during pilus assembly (Saulino et al., 1998, 2000).

Another important modification inherent in FimF, FimG, and FimA subunits is the presence of an amino (N)-terminal extension, which does not contribute to the Ig fold and remains disordered in crystal structures (Choudhury et al., 1999; Sauer et al., 1999). During pilus assembly, as subunits are presented to the usher, the N-terminal extension of the incoming subunit displaces the G1β strand of the chaperone that is occupying the cleft of the previous subunit, in a process termed "donor strand exchange" (Choudhury et al., 1999; Sauer et al., 1999). Interestingly, the N-terminal extension of the incoming subunit inserts into the cleft of the previous subunit in the classical, antiparallel orientation (Sauer et al., 2002). The atypical fold created by chaperone binding holds the pilin cleft in an open conformation. During donor strand exchange, insertion of the N-terminal extension induces a topological transition wherein the pilin cleft closes around the N-terminal extension to form the classical Ig fold. This presumably provides the driving force for pilus assembly (Sauer et al., 2002). Thus, ordered pilus assembly, carried out by donor strand complementation and donor strand exchange, results in numerous individual subunits incorporated into a single macromolecular structure, with each pilus subunit tightly bonded with the subunit immediately before and after (Figure 1B).

The FimH adhesin at the tip of a type 1 pilus comprises two domains: an N-terminal lectin domain, which acts as the primary adhesive component; and a C-terminal Ig-like pilin domain (Choudhury et al., 1999). Similar to other pilus subunits, the FimH C-terminal pilin domain is also missing its C-terminal β-strand, and donor strand complementation ensures its stability in the periplasm (Choudhury et al., 1999). The N-terminal lectin domain of FimH has an overall jellyroll-like topology formed by an 11-stranded β-barrel (Figure 3A) (Choudhury et al., 1999). The mannose binding site, which exists as a deep pocket lined by acidic amino acids, resides at the tip of this lectin domain (Choudhury et al., 1999; Hung et al., 2002). The acidic residues of this pocket are highly conserved across strains of *E. coli* that cause urinary tract infection, but several enterohemorrhagic *E. coli* strains have a mutation in this mannose binding site (Hung et al., 2002). Mutations in these pocket residues abolish binding to bladder cells, suggesting a mechanism for tropism of uropathogenic strains of *E. coli* for the urinary tract (Hung et al., 2002). Under shear stress, FimH has been shown to increase its binding affinity for the receptor (Thomas et al., 2002). Structural, biochemical, and immunohistochemical studies have shown that mono-mannose is the natural receptor for uropathogenic *E. coli* in the bladder (Figure 3A) (Hung et al., 2002). It appears, then, that uropathogenic strains of *E. coli* have evolved a FimH molecule that can take advantage of the bladder environment in order to achieve rapid colonization. Thus, uropathogenic strains of *E. coli* have optimized themselves for colonization of the urinary tract.

2.1.2. P Pili

Like type 1 pili, P pili are also vital for infection and colonization of the urinary tract, although P-piliated *E. coli* are more often associated with infection of the kidney (pyelonephritis) (Jones et al., 1996; Sauer et al., 2000). The PapG adhesin located at the tip of P pili binds to Galα(1–4)Gal residues on the globoseries of glycolipids present on the kidney epithelium (Dodson et al., 2001; Thanassi and Hultgren, 2000). PapG is encoded on the *pap* gene cluster, along with the major structural subunit of the pilus rod (PapA), a rod terminator involved in anchoring the pilus to the membrane (PapH), an outer membrane usher (PapC), a periplasmic chaperone (PapD), and adaptor and tip subunits (PapK, PapE, PapF) (Hung and Hultgren, 1998; Jones et al., 1996) (Figure 1A). In addition, located upstream of the *pap* operon are two genes encoding for regulators of P pilus expression (PapI and PapB), which carry out a form of phase variation (Hung and Hultgren, 1998). However, unlike the type 1 pilus system, phase variation of P pili is dependent upon DNA methylation patterns of sites within the *papI–papB* intergenic region, which affects binding of several regulatory proteins, including H-NS, catabolite activator protein, and leucine-responsive regulatory protein (van der Woude et al., 1996). The interplay between these elements determines whether pilus expression is turned on or off.

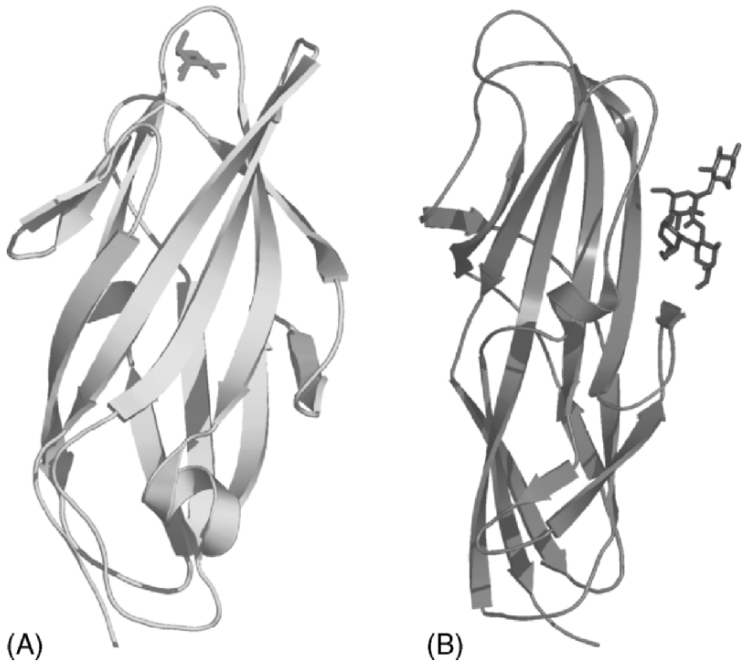

FIGURE 3. Crystal structures of the adhesin domains of FimH and PapG. (A) Ribbon representation of the FimH adhesin domain. This domain takes on a jellyroll-like topology, formed by an 11-stranded β-barrel. The FimH pilin domain and the FimC chaperone are not shown. The binding pocket, which sits at the tip of FimH, recognizes mannose residues. A mannose sugar is shown bound in this pocket. Structural coordinates from PDB ID 1KLF (Hung et al., 2002). (B) Ribbon representation of the PapG adhesin. The binding site on PapG is located on the side of the adhesin domain. Structural coordinates from PDB ID 1J8R (Dodson et al., 2001).

P pilus biogenesis is carried out in much the same way as type 1 pili (Choudhury et al., 1999; Hung and Hultgren, 1998; Jones et al., 1996; Sauer et al., 1999, 2000, 2002; Saulino et al., 2000; Thanassi et al., 1998b). However, several key differences exist between these systems relating to the mechanism of binding of P pili. First, the tip fibrillum, consisting of the PapG adhesin, the major tip protein PapE, and the adaptor proteins PapK and PapF, is much longer than the analogous fibrillum of type 1 pili (Jones et al., 1995). This is the result of incorporation of multiple PapE subunits into the P pilus structure (Jones et al., 1995, 1996). Another difference between type 1 pili and P pili is the location of the receptor binding site of the adhesin. Instead of being located at the tip, the binding site is oriented as a groove on the side of the lectin domain (Figure 3B) (Dodson et al., 2001). This means that the adhesin must lay on its side, parallel to its receptor, in order for binding to

occur. This process is facilitated by the longer tip fibrillum of P pili, which provides the increased flexibility needed in this region to facilitate the correct orientation of the adhesin for receptor recognition (Dodson et al., 2001). Thus, chaperone/usher pathways, while using highly homologous machinery, can produce structures with widely different activities and tropisms, depending upon the structural and biochemical properties of the adhesin.

2.2. Type IV Pili

Another adhesive organelle assembled by a variety of bacteria is the type IV pilus. Type IV pili are important for colonization of host tissues, biofilm formation, bacteriophage attachment, natural competence for DNA uptake, and a form of bacterial movement termed twitching motility (Davis and Waldor, 2003; DeVinney et al., 1999; Finlay and Falkow, 1997; Kaiser, 2000; Tonjum and Koomey, 1997). These processes are facilitated by a mechanism of pilus retraction, whereby the pilus is disassembled at the base (Kaiser, 2000; Wolfgang et al., 1998, 2000).

Type IV pilus biogenesis is quite distinct from the chaperone/usher pathway, with the type IV pilus machinery bearing homology to the type II system of bacterial secretion (Durand et al., 2003; Finlay and Falkow, 1997; Lory and Strom, 1997; Sauvonnet et al., 2000; Tonjum and Koomey, 1997; Wolfgang et al., 2000). In type IV pilus biogenesis, the organelle is built at the inner membrane and protrudes through the outer membrane as it grows (Soto and Hultgren, 1999; Wolfgang et al., 2000). These mechanisms utilize a pre-pilin peptidase, an outer membrane pore, and nucleotide-binding proteins to assist pilus assembly, as exemplified by the biogenesis of the type IV pilus of pathogenic *Neisseria* Spp. (Finlay and Falkow, 1997; Lory and Strom, 1997; Soto and Hultgren, 1999; Tonjum and Koomey, 1997; Wolfgang et al., 1998, 2000).

2.2.1. Type IV Pili of Pathogenic *Neisseria*

In pathogenic *Neisseria*, such as *N. gonorrhoeae*, type IV pilin genes are spread throughout the genome, as opposed to being confined to one locus (Tonjum and Koomey, 1997). The major structural subunit, PilE, has a highly conserved N-terminus with an unusual leader peptide, and a highly variable C-terminus (Finlay and Falkow, 1997; Tonjum and Koomey, 1997). This C-terminal variation results from homologous recombination between structural subunit genes and multiple silent alleles, which causes neisserial pili to undergo antigenic variation (Finlay and Falkow, 1997; Tonjum and Koomey, 1997). Following translocation into the periplasm, nascent pilin subunit monomers are tethered to the inner membrane by their N-terminal leader peptide (Lory and Strom, 1997; Soto and Hultgren, 1999; Tonjum and Koomey, 1997). A pre-pilin peptidase, PilD, located in the inner membrane, cleaves off this leader peptide and *N*-methylates the subunits, making them competent for assembly into the pilus filament (Lory and Strom, 1997) (Figure 4).

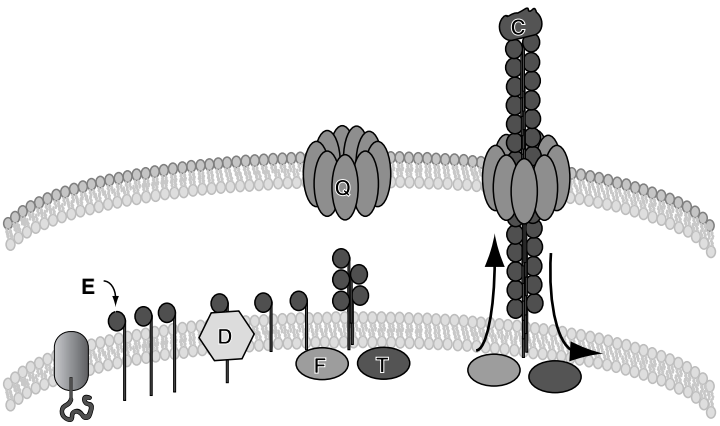

FIGURE 4. Biogenesis of neisserial type IV pili. After translocation into the periplasm, pilin subunits (PilE) are retained in the inner membrane by their unusual leader peptides. These leader peptides are cleaved by a pre-pilin peptidase, PilD. The conserved regions of PilE monomers interact to form a pilus rod rising from the inner membrane. This growing pilus rod traverses the bacterial outer membrane through a multimeric channel formed by PilQ. The interplay between the nucleotide binding proteins PilF and PilT modulates extension and retraction of the pilus, respectively. PilC is an adhesin that resides at the tip of neisserial type IV pili.

The crystal structure of the mature PilE monomer has been solved, and it has revealed clues as to the mechanisms of pilus biogenesis and antigenic variation (Parge et al., 1995). PilE takes on a ladle shape, with the highly conserved N-terminus forming an α-helix as the handle of the ladle, and the variable C-terminus forming the globular head (Parge et al., 1995) (Figure 5). Modeling studies have proposed that, as PilE monomers assemble into the pilus rod, their conserved N-terminal helices interact in the center of the pilus, while the variable C-terminal globular domains are oriented outward (Forest and Tainer, 1997; Parge et al., 1995). This may be equated with an extended flower arrangement where the stems (helices) form the core of the arrangement and the blossoms (globular heads) point out. The globular heads pack against each other to form a compact pilus structure, where conserved domains are buried in interactions with neighboring subunits and hypervariable regions are exposed on the surface of the pilus (Forest and Tainer, 1997; Parge et al., 1995) (Figure 4). The overall pilus structure depends on conserved amino acids that are involved in linking individual pilin monomers to adjacent subunits. This ensures the maintenance of pilus architecture despite antigenic variation. This pilus structural model also suggests a mechanism of immune evasion, whereby the conserved amino acid residues are hidden, while exposed residues are hypervariable and can change by antigenic variation.

FIGURE 5. Crystal structure of PilE. The α-helix of the PilE N-terminus forms the "handle" of a ladle shape. The C-terminal β-sheets form a globular head wherein hypervariable residues sit on the surface of the domain. As PilE monomers come together to form a pilus, the conserved N-termini interact in the center of the pilus, while the heads pack against each other to expose only the variable residues. Structural coordinates from PDB ID 1AY2 (Parge et al., 1995).

As the subunits are assembled, they are translocated across the outer membrane through PilQ, which forms a multimeric channel through the membrane (Tonjum and Koomey, 1997) (Figure 4). The bacterium can sense whether this channel is present, and mutants in *pilQ* do not assemble pili. However, in the presence of an additional mutation in another pilus gene, *pilT*, pili are assembled but form lateral aggregates enclosed in membranous protrusions from the bacterium (Wolfgang et al., 2000). These protrusions occur because the bacterium continues to assemble pili despite the loss of the outer membrane channel, and these pilus rods push against the outer membrane. PilT, and the homologous PilF, are nucleotide-binding proteins that reside in the inner membrane (Kaiser, 2000; Tonjum and Koomey, 1997; Wolfgang et al., 1998, 2000). While mutations in *pilT* lead to increased piliation, *pilF* mutants display abrogated pilus production (Wolfgang et al., 1998, 2000). These studies have revealed that PilF and PilT have opposite effects on pilus stability: PilF is required for multimerization of subunits and fiber formation, and PilT mediates fiber depolymerization (pilus retraction) and subunit degradation (Wolfgang et al., 2000) (Figure 4). This pilus retraction mechanism is important for the many functions of type IV pili, and specifically for twitching motility (Kaiser, 2000; Wolfgang et al., 1998). Thus, type

IV pilus biogenesis and functionality are dependent upon the interplay between PilF and PilT.

Binding activity of neisserial type IV pili to host cells is accomplished by phase variable expression of another pilus gene, *pilC*, which has been shown to be an adhesin (Rudel et al., 1995). Immunogold electron microscopy has shown that PilC is localized to pilus tips (Rudel et al., 1995). Once expressed, PilC mediates binding specifically to human epithelial cells, but not to human fibroblasts or cells of nonhuman origin (Rudel et al., 1995). This suggests that, as with the adhesins of the chaperone/usher pathway of pilus assembly, it is the adhesin of neisserial type IV pili that determines host and tissue tropism.

2.2.2. Toxin Coregulated Pilus of *Vibrio cholerae*

The toxin coregulated pilus (TCP) of *V. cholerae* is a type IV pilus that provides an intriguing glimpse at virulence regulation and pathogenic evolution. In addition to aiding bacterial colonization of the human intestine, it serves as the receptor for the cholera toxin bacteriophage (CTXΦ), which, upon infection, integrates into the genome of *V. cholerae* (Boyd et al., 2001; Davis and Waldor, 2003; Lee, 1999). Encoded on this phage genome are the genes for production of cholera toxin (CT), a major factor contributing to the diarrhea associated with the disease (Davis and Waldor, 2003). These genes are regulated by a complicated cascade involving the ToxRS two-component regulatory system, which activates expression of virulence regulatory genes such as *toxT* (on the TCP gene cluster), as well as the genes for CT, *ctxAB* (Boyd et al., 2001). Once expressed, ToxT can also activate production of these CT genes and the TCP locus (Boyd et al., 2001). Thus, toxin production and TCP expression are coregulated.

The TCP gene cluster, interestingly, has a significantly different guanine-cytosine (GC) content than the remainder of the genome, suggesting that this locus was acquired by horizontal gene transfer and remained in the genome as a pathogenicity-associated island (Davis and Waldor, 2003; Karaolis et al., 1999). Recently, it was proposed that this pathogenicity island is actually the genome of a bacteriophage, Vibrio Pathogenicity Island Phage (VPIΦ) (Karaolis et al., 1999). This suggests an evolutionary cascade of sequential infection with bacteriophages. First, infection with VPIΦ would have conferred upon *V. cholerae* the ability to produce the TCP, which is the receptor for CTXΦ. Subsequent infection with CTXΦ would create a CT-producing strain (Karaolis et al., 1999). It is intriguing to speculate as to whether other known type IV pilus apparatuses, or other adhesive structures, were acquired by other organisms in a similar manner.

2.3. Afimbrial Adhesive Structures

Many microorganisms produce adhesive molecules that do not associate with a pilus. These afimbrial adhesins often mediate more intimate interactions either with a target cell or between other bacterial cells. While there are

numerous afimbrial adhesive structures and pathways for adhesin display on the bacterial surface, some common themes have emerged.

2.3.1. Afimbrial Adhesins of *Escherichia coli*

Many strains of *E. coli* produce related afimbrial adhesins (AFA), grouped into the Dr adhesin family. This family connects several members on the basis of similar genetic structure and the common recognition of decay-accelerating factor (DAF) as a receptor (Nowicki et al., 2001). DAF has been found in the respiratory, urinary, genital, and digestive tracts, and Dr adhesins have been shown to be important for binding to numerous sites in the intestine and the urinary tract (Mulvey, 2002; Nowicki et al., 2001). Binding at these sites by Dr adhesins also enhances invasion into the epithelium (Mulvey, 2002). Although most members of the Dr adhesin family are afimbrial, biogenesis often involves the efforts of multiple gene products nevertheless, including a chaperone, an usher, and an adhesin (Hung and Hultgren, 1998; Nowicki et al., 2001). Although the AFA chaperones are related to those of pili assembled by the chaperone/usher pathway, these two chaperone groups differ slightly in their structural details. Pilus chaperones contain a short amino acid loop between their G1β strand (the strand donated during donor strand complementation to stabilize pilus subunits) and the previous strand (Figure 2). In contrast, AFA chaperones possess a longer loop between these two critical strands (Hung and Hultgren, 1998). Because of this longer loop, more contacts are made between the chaperone and the pilus subunit (Zavialov et al., 2003). It is important to note that chaperones with a shorter loop direct assembly of pilus structures, but those with a longer loop produce afimbrial structures (Hung and Hultgren, 1998). Thus, slight alterations in the assembly process can produce widely divergent adhesive structures.

2.3.2. Autotransporter Adhesins

Autotransporters form a large family of secreted molecules produced by a variety of unrelated microorganisms, including species of *Rickettsia*, *Bordetella*, *Neisseria*, *Helicobacter*, and many members of the family *Enterobacteriaceae*, among others (Henderson and Nataro, 2001; Henderson et al., 1998). Various autotransporter proteins have been shown to function as proteases, toxins, invasins, motility mediators, serum resistance factors, or adhesins (Henderson and Nataro, 2001; Henderson et al., 1998). Adhesive autotransporters often contain an RGD amino acid motif, which is associated with binding integrin molecules on mammalian cells (Henderson et al., 1998). However, not all autotransporter adhesins possess this motif, and thus they bind to host cells in a different fashion (Fink et al., 2003; Henderson and Nataro, 2001; Henderson et al., 1998). In order to mediate their functions, including host cell binding, all autotransporters must be presented on the surface of the bacterium.

Outer membrane translocation of autotransporters is unique among all secreted proteins in that the machinery needed to carry out this transport is

part of the molecule itself (Henderson and Nataro, 2001; Henderson et al., 1998). The nascent molecule contains three functional domains: an N-terminal signal sequence, the passenger domain exhibiting the functional properties of the molecule, and the C-terminal β-domain (Henderson and Nataro, 2001; Henderson et al., 1998) (Figure 6). Upon expression, the signal sequence targets the molecule for export to the periplasm by the general secretory pathway. Once in the periplasm, hydrophobic amino acids mediate insertion of the C-terminal β-domain into the outer membrane to form a pore. This pore is composed of 10–18 amphipathic β-sheets that align antiparallel to each other to create a β-barrel with a central hydrophilic channel. The passenger domain travels through this outer membrane β-barrel pore and becomes exposed on the extracellular surface of the bacterium (Henderson and Nataro, 2001; Henderson et al., 1998) (Figure 7A). At this point, passenger domains meet one of three fates. Some are cleaved off, either autocatalytically or by another protease, and are released from the bacterium. Others are cleaved, but stay associated with the β-domain or the outer membrane. Finally, many passenger domains are never cleaved, and these remain as part of the autotransporter, tethered to the membrane and displayed on the surface (Henderson and Nataro, 2001; Henderson et al., 1998).

Adhesin autotransporters typically need to stay associated with the outer membrane in order to mediate binding of host cells. However, some, such as the Hap autotransporter of *Haemophilus influenzae*, can be cleaved and released from the bacterium (Fink et al., 2003; Henderson and Nataro, 2001; St Geme, 2002). *H. influenzae* colonizes the respiratory tract and can cause several disease manifestations, including otitis media, sinusitis, bronchitis, and pneumonia (Fink et al., 2003; St Geme, 2002). Hap, one of many adhesive molecules produced by this organism, has been shown to bind to cultured human respiratory cells as well as to laminin, fibronectin, and collagen IV, which are all components of the mammalian extracellular matrix (Fink et al., 2003; St Geme, 2002). The C-terminal portion of the Hap passenger domain mediates these binding activities and also promotes bacterial

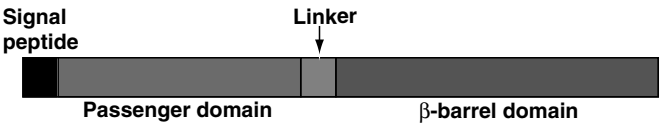

FIGURE 6. Domain structure of autotransporter proteins. Autotransporter proteins contain a signal peptide at their N-terminus to direct secretion to the periplasm by the general secretory pathway. Following the signal peptide is the passenger domain, which, when extracellular, serves at the interactive component of the autotransporter. In the case of autotransporter adhesins, the passenger domain forms the adhesive element. A short linker connects the passenger domain to the C-terminal domain, which forms a β-barrel pore of amphipathic β-sheets in the outer membrane.

aggregation (Fink et al., 2003). However, the N-terminus contains a serine protease catalytic site that autoproteolytically releases the passenger domain into the extracellular environment (Fink et al., 2003; Henderson and Nataro, 2001; St Geme, 2002). This might allow the bacteria to better evade the immune system or enhance colonization by facilitating spread from the initial site of attachment (Henderson and Nataro, 2001). Furthermore, the serine protease activity of Hap might be involved in tissue destruction or other pathogenic events (Henderson and Nataro, 2001). It is interesting that secretory leukocyte protease inhibitor, present in respiratory secretions and upregulated upon infection, abrogates autoproteolysis of Hap (Fink et al., 2003; Henderson and Nataro, 2001; St Geme, 2002). Thus, the local environment seems to influence the proteolytic and adhesive activities of this multifunctional autotransporter protein. More than likely, other adhesins are also influenced by their environment as they promote adherence, colonization, and spread during pathogenesis.

2.3.3. Two-component Secretion

Similar to autotransporter proteins, two-component secretion consists of a passenger molecule translocated into the extracellular environment through a β-barrel pore in the outer membrane (Jacob-Dubuisson et al., 2001). However, the outer membrane pore domain is a separate molecule from the secreted protein, these two components forming an "unlinked" autotransport system (Figure 7B) (Jacob-Dubuisson et al., 2001; St Geme, 2002). Like the autotransporters, the growing two-component secretion family of exoproteins perform a variety of functions, including adhesion.

One well-studied adhesin of this family is the filamentous hemagglutinin (FHA) of *Bordetella pertussis* (Jacob-Dubuisson et al., 2001; Locht et al., 2001). FHA, the major adhesin of *B. pertussis*, forms an extremely large 500 Å-long, 40 Å-wide filament (Kajava et al., 2001; Locht et al., 1993). Despite its size, FHA is abundantly expressed by *B. pertussis* and is important for binding to the respiratory epithelium (Kajava et al., 2001; Locht et al., 2001). The *fha* locus, regulated by the BvgAS two-component system in response to environmental cues, encodes for the structural protein FhaB and the outer membrane β-barrel protein FhaC (Jacob-Dubuisson et al., 2001; Locht et al., 1993, 2001). FhaB begins in the cytoplasm as a 367 kDa polypeptide and is processed during secretion to 220 kDa, the result of cleavage of the signal peptide and the C-terminal third of the protein (Kajava et al., 2001; Locht et al., 1993). FhaB is translocated across the outer membrane in an extended conformation through FhaC (Locht et al., 2001). Once extracellular, FhaB folds into a conformation rich in β-structure, probably as a β-helix (Kajava et al., 2001). This mature FHA structure contains several features important for binding host cell receptors in the respiratory epithelium, including an RGD motif for binding to integrins (specifically to complement receptor 3), a heparin binding site for recognizing sulphated glycolipids on host cells, and

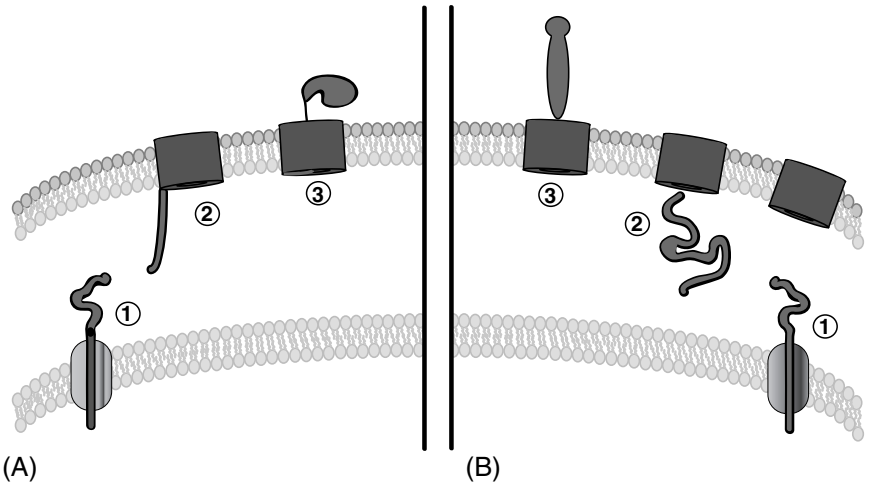

FIGURE 7. Secretion of autotransporters and two-component secretion. (A) The secretion mechanism of autotransporter proteins. After translocation into the periplasm through the general secretory pathway (1), the autotransporter migrates to the outer membrane. The C-terminus of the molecule inserts into the outer membrane (2), forming a β-barrel pore of amphipathic β-sheets. The passenger domain travels through this pore (3) to reach the extracellular surface. Hence, a single autotransporter possesses the necessary machinery to carry out its own secretion and surface display. Once displayed on the bacterial surface, autotranporter adhesins may mediate binding to host cell receptors. (B) The two-component secretion scheme. Two-component secretion is quite similar to autotransporter secretion. However, the passenger domain and the outer membrane pore are two separate molecules. For adhesive molecules secreted by this pathway, the adhesin is translocated into the periplasm through the general secretory pathway (1). This molecule then crosses the periplasm to an outer membrane pore (2). Finally, the adhesin is translocated across the pore (3) and forms the mature adhesin on the extracellular surface.

various carbohydrate recognition domains (Kajava et al., 2001; Locht et al., 2001). Structural analyses indicate that these sites are all on one face of the FHA filament and suggest that FHA might form a bridge between *B. pertussis* and its target cells, with one side of FHA interacting with the microorganism and the other side binding to host cell receptors (Kajava et al., 2001). In this way, FHA promotes disease by maintaining contact with both the pathogen and the host. Thus, FHA is another example of an adhesive structure performing multiple functions in pathogenesis and disease.

2.4. Gram-positive Adhesins

Gram-positive organisms also produce a variety of adhesins. However, because of their lack of an outer membrane and the presence of a thick peptidoglycan layer, these microbes use methods different than Gram-negative

organisms to produce and display adhesive structures on their surface. There are several ways Gram-positive bacteria attach proteins to their cell wall, including noncovalent interactions with teichoic acids, attachment to the membrane by lipoproteins, and insertion of hydrophobic amino acid stretches into the membrane (Cabanes et al., 2002; Navarre and Schneewind, 1999). The most widespread method of surface protein expression is via covalent linkage of the molecule to the cell wall peptidoglycan in a process termed sorting (Navarre and Schneewind, 1999). In this scheme, secreted proteins contain a C-terminal hydrophobic domain and a charged tail, preceded by an LPXTG amino acid motif. Upon secretion across the membrane, the protein is retained in the membrane by the hydrophobic domain. An enzyme called a sortase then cleaves the LPXTG motif between the threonine and the glycine and covalently bonds the threonine to the growing peptidoglycan layer of the cell wall (Navarre and Schneewind, 1999). In this way, secreted molecules, including adhesins, can be displayed on the cell surface, waiting for interactions with ligands.

2.4.1. Internalin

The Gram-positive organism *Listeria monocytogenes*, a food-borne pathogen that can cause gastroenteritis, septicemia, and meningitis, among other clinical manifestations, produces a surface-presented adhesive molecule called internalin (Braun and Cossart, 2000; Cabanes et al., 2002). Internalin, tethered to the cell wall by the LPXTG mechanism, mediates binding and invasion of epithelial cells by recognizing E-cadherin present on the host epithelium (Braun and Cossart, 2000; Cabanes et al., 2002). In addition to the LPXTG motif, and a signal peptide for secretion, the internalin molecule, InlA, also contains two regions of amino acid repeats and a conserved inter-repeat region (IR) (Braun and Cossart, 2000; Cabanes et al., 2002) (Figure 8A). The N-terminal repeat region consists of a large number of leucine residues, forming a leucine-rich repeat (LRR) domain (Braun and Cossart, 2000; Cabanes et al., 2002) (Figure 8A). Such LRR domains are known to be involved in protein–protein interactions such as adhesion and receptor-ligand binding (Cabanes et al., 2002). Functional studies of InlA have shown that the LRR, as well as the IR region, are important for internalin activities, with the LRR recognizing E-cadherin and the IR region influencing proper folding of the molecule (Braun and Cossart, 2000). Crystal structures of the highly homologous InlB and InlH revealed a hydrophilic N-terminal calcium-binding cap followed by an elongated bowed tube structure of alternating β-strands and α-helices formed by the LRR (Marino et al., 1999; Schubert et al., 2001). The concave face of this curved region contains a cluster of hydrophilic amino acid residues and another cluster of negatively charged residues that most likely mediate interactions with the host receptors. This packing of interactive surfaces to one face of the adhesin is similar to the FHA-binding scheme, and may represent another common theme in

adhesin–receptor interactions. In addition, the N-terminal cap of InlB and InlH binds two calcium ions, and this is thought to be involved in assisting recognition of the receptor by forming a bridge between the bacterial molecule and the host receptor (Marino et al., 1999). The IR region folds into an Ig-like domain at the base of the molecule, which may be important for capping the LRR curve, or for heterophilic interactions with the Ig fold of E-cadherin (Schubert et al., 2001). InlA binding of E-cadherin, using these various structural features, is dependent upon a proline residue at position 16 in the E-cadherin structure (Braun and Cossart, 2000). Interestingly, mouse E-cadherin, while highly homologous with the human form, possesses a glutamic acid instead of proline at position 16, and internalin does not recognize this mouse molecule (Braun and Cossart, 2000). Thus, as with other adhesins, the structural properties of internalin and E-cadherin influence host and tissue tropism of the microorganism and specific interactions between adhesin and receptor.

2.4.2. M Protein of Group A *Streptococci*

Another LPXTG sorted surface molecule is M protein of group A streptococci (Navarre and Schneewind, 1999). This hypervariable adhesin divides group A streptococci into more than 80 serotypes, with different M serotype groups forming the basis of tropism for specific disease states (Cunningham, 2000). The numerous M protein variants recognize a multitude of ligands in the host plasma membrane and the extracellular matrix, including albumin, fibrinogen, fibronectin, IgG, kininogen, and plasminogen, as well as several complement regulatory molecules, such as factor H, FHL-1, and membrane cofactor protein (CD46) (Cunningham, 2000; Navarre and Schneewind, 1999). M proteins are composed of several repeat regions, dividing the molecule into an N-terminal variable domain and a C-terminal conserved domain (Courtney et al., 2002; Cunningham, 2000) (Figure 8B). A hypervariable region is located at the tip of the N-terminus. These domains form one long α-helix, and M protein is displayed as a coiled-coil dimer on the bacterial surface (Courtney et al., 2002; Cunningham, 2000). Many binding interactions between group A streptococci and host cells are mediated by the variable region at the N-terminus of M proteins. However, during skin infections, the repeat regions of the conserved domain bind to CD46 on keratinocytes (Courtney et al., 2002; Cunningham, 2000). A two-step binding process has been suggested in which initial, weak interactions between the microorganism and the host tissue are mediated by lipoteichoic acid on the bacterial surface. This paves the way for intimate, specific, and high-affinity associations of M proteins with host receptors (Courtney et al., 2002; Cunningham, 2000). In this manner, group A streptococci can form specific interactions with a variety of host receptors depending upon their M protein serotype.

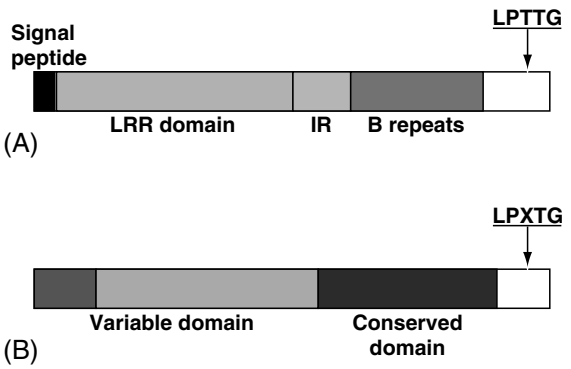

FIGURE 8. Domain structures of InlA and M protein. (A) The domain structure of InlA (internalin) of *Listeria monocytogenes*. InlA contains an N-terminal signal peptide, which directs secretion across the membrane. An LPTTG motif on the C-terminus ensures recognition of InlA by sortase and covalent linkage to the peptidoglycan layer of the cell wall. Additionally, InlA contains two repeat regions, the leucine-rich repeats (LRRs) and the B repeats, which are connected by the interrepeat region. Structural studies suggest that the LRRs form a bowed tube with a calcium-binding cap, and the IR forms an Ig-like fold at the base of the LRRs. (B) The domain structure of M protein of group A streptococci. M protein contains an LPXTG motif for covalent linkage to the cell wall by sortase. Several repeat regions divide M proteins into an N-terminal variable domain and a C-terminal conserved domain. The extreme N-terminus contains a hypervariable region.

2.5. Other Adhesins

Most Gram-positive organisms, as well as some Gram-negative bacteria, bind to various elements of the mammalian extracellular matrix. This recognition is carried out by adhesive molecules on the bacterial surface, termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) (Joh et al., 1999; Navarre and Schneewind, 1999). During infection, these MSCRAMM molecules bind to host matrix components such as fibronectin. Fibronectin also adheres to integrin molecules on host cells and thus forms a bridge between the bacterium and the host (Joh et al., 1999). This clever mechanism allows the infecting pathogen to recognize common matrix molecules in order to potentially adhere to a number of target locations.

Finally, many common surface elements of bacteria that typically provide other functions can also act as adhesins. Structures such as teichoic acids, lipoteichoic acids, flagella, and polysaccharide capsules have been shown to be involved in adherence of a variety of microorganisms to host tissues (Cunningham, 2000; Nougayrede et al., 2003; St Geme, 2002; Wilson et al., 2002). Often, these interactions are nonspecific, and they pave the way for more intimate interactions between specific adhesins and host cell receptors.

3. Consequences of Adhesion

Bacterial binding to host cells is an active event, and adhesion typically represents just the first step in microbial infection. Recognition and binding of a host receptor by adhesin leads to activation of complicated signaling pathways in both the bacterium and the host, potentially resulting in numerous downstream events related to pathogenesis of the microorganism. These events, such as colonization, biofilm formation, and invasion of host tissues, can lead to tissue destruction, enhance transmission of the organism, and even promote microbial persistence within the host. Binding to specific host tissues allows the bacterium to focus its efforts towards one site and provides direction for continued disease progression. In this way, bacterial attachment by means of adhesins represents the gateway for pathogenesis.

3.1. Activation of Bacterial Signaling Pathways

The initial binding event between the bacterial adhesin and its host receptor often signals the microbe that it has reached its target site and encourages the proper production of factors that propagate the infection. Likewise, signaling pathways in the bacterium may also guide the construction of the adhesive organelles themselves. In *E. coli*, assembly of the PapG adhesin into P pili is closely monitored by regulatory pathways such as the CpxRA two-component signal transduction pathway (Jones et al., 1997; Lee et al., 2004). The CpxRA pathway is fine-tuned to respond to extracytoplasmic stresses (Connolly et al., 1997; Cosma et al., 1995; Danese and Silhavy, 1997; Raivio and Silhavy, 1999), including alkaline pH (Nakayama and Watanabe, 1995), the buildup of misfolded or aggregated proteins (Jones et al., 1997), overproduction of the outer membrane protein NlpE (Snyder et al., 1995), and changes in inner membrane lipid composition (Mileykovskaya and Dowhan, 1997). During pilus biogenesis, a portion of PapG adhesins and some pilus subunits fail to interact with the chaperone and are driven OFF-pathway (Hung et al., 2001; Jones et al., 1997; Lee et al, 2004). These OFF-pathway events trigger the stimulation of the CpxRA pathway (Jones et al., 1997). Activation of Cpx is beneficial to the proper presentation of the adhesin, in part, because the pathway regulates the expression of envelope factors that aid in pilus biogenesis by functioning in protein folding and in degradation of misfolded and aggregated proteins (Hung et al., 2001; Jones et al., 1997; Raivio and Silhavy, 1999). These factors include the disulfide oxidoreductase DsbA (Connolly et al., 1997), peptidyl prolyl isomerases PpiA and PpiD (Dartigalongue and Raina, 1998; Pogliano et al., 1997), and the periplasmic protease DegP (Danese et al., 1995). The CpxRA pathway also positively affects the phase variation of the *pap* operon, which contains potential CpxRA consensus binding sequences upstream of its promoter (Hung et al., 2001). In this way, *E. coli* can maintain tight control over the timing and proper presentation of the PapG adhesin (Hung et al., 2001; Jones et al., 1997).

Increasing evidence is emerging which suggests that the CpxRA pathway may also play an important role in the pathogenesis of the bacteria. Consensus CpxRA binding sites have been found upstream of virulence factors such as hemolysin and cytotoxic necrotizing factor-1 in *E. coli* (Hung et al., 2001). Thus, just as adhesin–receptor interactions may initiate signaling cascades in the host cell, the adhesin may also signal the bacterial cell to express factors important in infection. Homologues of the CpxRA pathway have also been found in other bacteria, such as *Shigella* Spp. and *Salmonella typhimurium* (Nakayama and Watanabe, 1995; Suntharalingam et al., 2003). It will be interesting to determine if other adhesins assembled via the chaperone/usher pathway also activate two-component signal transduction systems in a similar manner.

3.2. Colonization of the Host

Bacterial colonization typically involves a large number of bacteria binding to numerous sites within the target tissue. Activation of bacterial signaling processes upon binding further enhances the ability of the bacteria to spread and disseminate from the initial site of binding. Host signaling pathways are also turned on by the binding interaction, and often it is the colonizing bacteria that actively stimulate host cascades. Enteropathogenic *E. coli* (EPEC) is an example of how a pathogen can skillfully manipulate the host epithelium in order to create close contact and tight binding. EPEC is a major cause of diarrheal disease in developing countries. During pathogenesis, aggregates of EPEC adhere as microcolonies to the intestinal epithelium in a process called localized adherence (DeVinney et al., 1999; Nougayrede et al., 2003). This initial colonization quickly leads to loss of microvilli on the intestinal epithelial cells at the site of attachment, forming plaques termed attaching and effacing (A/E) lesions (DeVinney et al., 1999; Nougayrede et al., 2003). The most striking phenotype of A/E lesions is the construction of extended actin-rich pedestals, produced by the infected intestinal cell underneath each bacterium (DeVinney et al., 1999; Nougayrede et al., 2003). These pedestals, created by massive actin polymerization underneath the bacterium, lifts the organism up into the intestinal lumen. The bacteria on the pedestals bind to the host cell membrane with as little as 10 nm distance in between, creating an intimate interaction between pathogen and host and accounting for the ability of EPEC to resist flow and shear forces encountered in the intestine (Nougayrede et al., 2003). Indeed, the effect of shear forces on bacterial adherence, invasion, and pathogenesis has recently become an area of increasing interest (Nickerson et al., 2004; Thomas et al., 2002, 2004).

Adhesins and adhesive structures play a vital role in the progression of EPEC diarrheal disease. EPEC produce a type IV pilus known as the bundle-forming pilus (BFP) due to the ability of the pili to bundle together into rope-like aggregates (Nougayrede et al., 2003). The genes encoding for this pilus are encoded on a large plasmid harbored in EPEC. BFP mediates initial contact

with the intestinal epithelium, resulting in localized adherence (Goosney et al., 1999). Alternatively, it has been suggested that BFP is primarily involved in interbacterial aggregation and microcolony formation, and that other adhesive structures, such as flagella, initiate the first contacts between EPEC and the intestine (DeVinney et al., 1999; Nougayrede et al., 2003). In either case, BFP is crucial for formation of microcolonies and localized adherence (Nougayrede et al., 2003). Soon after these initial interactions, the bacteria, now close to the cell, extend a type III secretion apparatus, which is encoded on a chromosomal pathogenicity island known as the locus of enterocyte effacement (LEE) (DeVinney et al., 1999; Nougayrede et al., 2003) (Figure 9A). The LEE also encodes for several effector molecules, involved in effacement of intestinal microvilli, and the adhesin intimin, which is found in the outer membrane of the microorganism (Nougayrede et al., 2003). The N-terminal domain of intimin forms a β-barrel in the bacterial outer membrane, while the C-terminus extends out from the bacterium with three Ig-like domains and a distal lectin domain (Nougayrede et al., 2003). One of the main functions of the type III secretion system (T3SS) during EPEC pathogenesis is the translocation of the receptor for intimin, Tir (for translocated intimin receptor), directly into the cytoplasm of the intestinal cell (Campellone and Leong, 2003; DeVinney et al., 1999; Nougayrede et al., 2003). After translocation, Tir inserts into the intestinal cell cytoplasmic membrane, allowing interaction between intimin and Tir, and consequently the docking of EPEC onto the host cell. Multiple intimin/Tir binding events along the bound edge of the bacterium leads to the intimate association of the pathogen with the epithelium (Campellone and Leong, 2003; DeVinney et al., 1999; Nougayrede et al., 2003). Soon after translocation and incorporation into the host cell membrane, the cytoplasmic domains of Tir become phosphorylated and signal actin polymerization by recruiting Nck, N-WASP, and Arp2/3 (Campellone and Leong, 2003). This actin polymerization leads to the formation of pedestals, lifting tightly bound bacteria up from the infected cell into the intestinal lumen (Campellone and Leong, 2003) (Figure 9A). In this way, EPEC efficiently manipulates its target cell to maintain tenacious association with the epithelium and colonize the intestine. Further, the mechanism of following weak interactions with intimate adherence, similar to M protein colonization during skin infections, may very well be another common adhesive strategy.

3.3. Biofilm Formation

In the environment, many bacterial species form specialized microbial communities called biofilms. Generally, biofilm formation initiates with binding to a surface, followed by bacterial aggregation and differentiation. The bacteria encase themselves in a polysaccharide matrix, which scavenges and traps nutrients from the environment as well as provides protection for the bacteria (Dunne, 2002). Within this matrix, the bacteria differentiate to display

altered growth rates and gene transcription phenotypes. Regional differentiation establishes distinct subpopulations of bacteria that carry out unique activities to benefit the entire community (Donlan and Costerton, 2002; Dunne, 2002). Biofilms have also been found to be associated with bacterial infections (Donlan and Costerton, 2002; Dunne, 2002). Such *in vivo* biofilms protect the bacteria from immune system clearance and confer increased resistance to antibiotics (Donlan and Costerton, 2002; Dunne, 2002).

The Gram-negative opportunistic pathogen *Pseudomonas aeruginosa* is proficient at creating biofilms in the lungs of cystic fibrosis patients (Donlan and Costerton, 2002). Once this biofilm is established, the infection is nearly impossible to clear, allowing the bacteria to persist and establish chronic infection despite potent immune defenses and repeated courses of antibiotics (Davies, 2002). Initial colonization appears to take place with nonmucoid strains. However, due to environmental cues or other poorly understood stimuli, the bacteria convert to a mucoid phenotype, producing large quantities of exopolysaccharides called alginate (Donlan and Costerton, 2002). Studies on *in vitro P. aeruginosa* biofilms have shown that flagella and type IV pili are important for biofilm formation (O'Toole and Kolter, 1998). First, individual *P. aeruginosa* use flagella to bind to a surface. Then, twitching motility, carried out by the extension and retraction of type IV pili, allows the bacteria to migrate toward each other and form microcolonies on the bound surface. Finally, production of alginate encases the bacteria, creating a protective environment wherein the bacteria can survive in the face of severe insults from the environment or the host (Dunne, 2002). *P. aeruginosa* forms biofilms in the lungs of approximately 80% of patients with cystic fibrosis, and many efforts are underway to develop therapeutics to disrupt biofilms and inhibit biofilm formation in order to treat these antibiotic-resistant, chronic infections (Davies, 2002; Donlan and Costerton, 2002). It is likely that therapies targeting flagella, type IV pili, and other adhesive structures of *P. aeruginosa* will be beneficial in preventing colonization and biofilm formation.

3.4. Bacterial Invasion of Host Tissues

Many bacterial pathogens invade into the host epithelium or other host tissues in order to evade the immune system or find nutrients. Once inside the host cell, microorganisms may multiply at this primary site and cause localized tissue damage, or they may disseminate systemically through lymph nodes or the bloodstream to reach locations far from their initially bound tissue. Some bacteria forcibly invade by directly causing focused damage to the host cell membrane (Finlay and Falkow, 1997). However, a common theme in bacterial invasion of host epithelium involves bacterial manipulation of host signaling pathways to generate major cytoskeletal rearrangements in the host cell, similar to EPEC-induced pedestal formation (Campellone and Leong, 2003; Finlay and Falkow, 1997). Often, the adhesive molecule of the bacterium also mediates this invasion. For example, internalin of *L. monocytogenes* not only

binds to the host intestinal epithelium but also induces cytoskeletal changes in its bound cell, activating host processes through stimulation of the signaling component of its bound E-cadherin (Braun and Cossart, 2000; Finlay and Falkow, 1997). The epithelial cell membrane protrudes and zippers around the bound bacterium, eventually completely engulfing and internalizing the pathogen (Braun and Cossart, 2000; Finlay and Falkow, 1997) (Figure 9B).

This induced phagocytosis into nonphagocytic cells is carried out by several other intestinal pathogens, such as enteropathogenic species of *Yersinia*, *Salmonella*, and *Shigella*, all of which stimulate diarrhea production in the infected host (Brumell et al., 1999; Goosney et al., 1999; Isberg and Barnes, 2001; Vazquez-Torres and Fang, 2000). *Yersinia enterocolitica* and *Y. pseudotuberculosis* also invade into the intestinal epithelium via a zipper mechanism (Figure 9B). These pathogens both encode a surface exposed protein called invasin, which recognizes and binds to β1 integrins on the surface of intestinal M cells (Isberg and Barnes, 2001; Vazquez-Torres and Fang, 2000). Other adhesins that aid binding of *Yersinia* to M cells include Ail protein, the pH6 antigen, and the YadA protein (Vazquez-Torres and Fang, 2000). Invasin contains an N-terminal domain important for outer membrane localization, followed by four Ig-like domains, and a C-terminal lectin domain fused with the fourth Ig-like domain (Isberg and Barnes, 2001). These features display sequence homology and structural similarity with EPEC intimin (Isberg and Barnes, 2001). Clustering of β1 integrins on the M cell, brought about by multiple invasin–integrin interactions and invasin dimerization, signals restructuring of the host cytoskeleton and zippering of the pathogen into the intestinal cell (Isberg and Barnes, 2001). *Yersinia* then migrates through the M cell, and piggybacks on phagocytic cells to regional lymph nodes, where the pathogen gains access to disseminate systemically (Isberg and Barnes, 2001; Vazquez-Torres and Fang, 2000).

Salmonella and *Shigella* also recognize and invade into intestinal M cells, but the mechanism of their invasion is quite distinct from that of enteropathogenic *Yersinia*. Instead of membrane protrusions zippering around and engulfing the bound pathogen, M cells bound by *Salmonella* and *Shigella* undergo severe membrane ruffling, and the invasion event occurs as a "splash," with the bacteria taken up in a macropinocytosis-like manner (Brumell et al., 1999; Finlay and Falkow, 1997; Goosney et al., 1999; Vazquez-Torres and Fang, 2000). The pathogens induce this activity by using a T3SS to inject effector molecules directly into the M cell cytoplasm (Brumell et al., 1999; Vazquez-Torres and Fang, 2000). These effector molecules stimulate signaling pathways that lead to actin rearrangement, similar to the molecules secreted by the T3SS of EPEC although with drastically different results (Brumell et al., 1999; Campellone and Leong, 2003; Goosney et al., 1999; Vazquez-Torres and Fang, 2000) (Figure 9C). Once inside the M cell, *Salmonella* can travel to lymph nodes, via parasitized dendritic cells, to establish systemic infection, while *Shigella* prefers to remain in the intestinal epithelial cells (Goosney et al., 1999; Vazquez-Torres and Fang, 2000). *Shigella* then

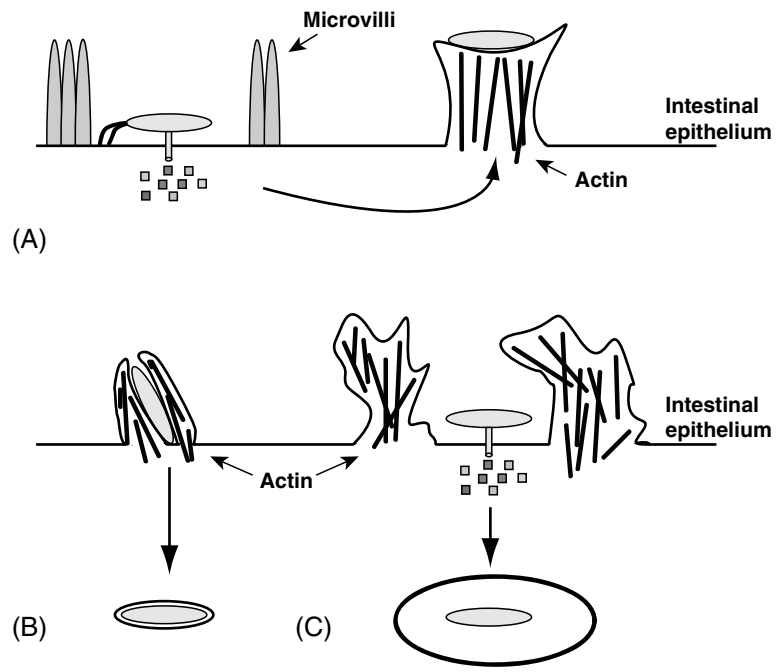

FIGURE 9. Bacterial manipulation of host cell signaling. (A) EPEC colonization of intestinal epithelium and pedestal formation. EPEC bind to the intestinal epithelium by means of the BFP, which leads to attachment of the bacteria and effacement of intestinal microvilli. EPEC then extend a type III secretion apparatus to inject effector molecules directly into the host cell cytoplasm. One of these molecules, Tir, inserts into the host cell cytoplasmic membrane and acts as the receptor for intimin, displayed on the bacterial surface. Binding of intimin to Tir leads to intimate adherence of EPEC onto the intestinal epithelial cell. Phosporylation of Tir in the host cell cytoplasmic membrane activates intestinal cell signaling to recruit actin to the site of bacterial attachment. Massive actin polymerization leads to formation of actin-rich pedestals, which lift the bacteria up into the intestinal lumen. (B) Zipper-like internalization of bacteria into host cells. Bacterial binding to their target cells induces actin polymerization immediately around the bacterium and a "zippering" of the membrane around the microorganism. The bacterium is completely engulfed and internalized into the host cell. During infection with *Listeria monocytogenes*, binding of InlA to E-cadherin stimulates the cytoplasmic signaling component of E-cadherin to initiate these cytoskeletal rearrangements. Enteropathogenic *Yersinia* are also internalized by this mechanism as the protein invasin binds and clusters β1 integrins. Clustering of integrins leads to host cell signaling and cytoskeletal rearrangements. (C) Membrane ruffling and internalization induced by *Salmonella* and *Shigella*. These pathogens bind to M cells and inject effector molecules through a type III secretion apparatus. These effector molecules stimulate major rearrangements of the host cell cytoskeleton that appear as ruffling of the membrane. Membrane ruffles surround the bacteria and eventually close around the microorganisms, internalizing them by a macropinocytosis-like mechanism.

infects adjacent intestinal cells through the unusual mechanism of actin-based motility, whereby the pathogen generates propulsion by polymerizing actin at one pole (Goosney et al., 1999; Vazquez-Torres and Fang, 2000). The preferential recognition of M cells by *Salmonella* and *Shigella* suggests that they produce specific adhesins that bind to receptors on these cells. Indeed, *Salmonella* use long polar pili to target themselves to M cells (Baumler et al., 1996). However, these interactions, along with the interactions of *Shigella* with M cells, remain ill-defined. It is interesting, though, that *Shigella* prefer to invade M cells of the colorectal mucosa, while *Salmonella* invades M cells in the distal small intestine (Vazquez-Torres and Fang, 2000). To account for this tropism, these two similar microorganisms quite likely produce different adhesins, which recognize slight variations in the content of surface molecules on M cells at these two locations. Thus, invasion into the intestinal epithelium is mediated by various microorganisms using different mechanisms of invasion, but with similar consequences for the host: intracellular bacteria that take over this niche for their own purposes.

3.5. *Uropathogenic* Escherichia coli *Pathogenesis*

An excellent model system in which to study adhesin–receptor interactions is the pathogenesis of uropathogenic *E. coli* (UPEC) in the urinary tract. UPEC account for 70–95% of all community-acquired urinary tract infections (UTIs), and are associated with a majority of nosocomial UTIs (Hooton and Stamm, 1997). UTIs afflict a large percentage of women, resulting in large economic costs for treatment and diagnosis (Foxman, 1990, 2002; Patton et al., 1991). The most common UTI manifestations are infection of the bladder (cystitis) and the kidney (pyelonephritis), leading to many symptoms, including painful, frequent, and urgent urination; lower back pain; and suprapubic tenderness (Hooton and Stamm, 1997). Although thought of as acute and self-limiting, UPEC-induced UTIs tend to recur at an incredibly high rate, with over one-fourth of women with a primary occurrence experiencing at least one recurrence within 6 months (Foxman, 1990). To accomplish these clinical presentations, UPEC express numerous, multifunctional adhesins, which allow the bacteria to colonize several different locations in the urinary tract as well as promote further host and bacterial signaling. Recent genomic analysis has revealed that UPEC strains may encode for at least two type IV pilus structures, several different adhesive autotransporters, and at least 12 different chaperone/usher pilus gene clusters, including type 1 pili and P pili (Welch et al., 2002). Further, some UPEC strains are known to express several Dr family afimbrial adhesins (Mulvey, 2002; Nowicki et al., 2001). It is likely that further genomic analysis and comparisons between strains will reveal many other adhesins important for pathogenesis of UPEC in UTIs.

Animal models of UTI have elucidated a pathogenic cycle whereby UPEC infects the bladder and subverts host defenses to create a chronic infection.

Pathogenesis begins with introduction into the bladder and binding to the superficial bladder epithelium via recognition of epithelial uroplakins by FimH on the tip of type 1 pili (Mulvey et al., 1998). Uroplakins are mannosylated membrane proteins found coating the surface of the bladder epithelium that strengthen this epithelial barrier and form an impermeable shield preventing diffusion of toxins and other chemicals into bladder tissues (Hu et al., 2002; Lewis, 2000). Binding of type 1 pili to these mannosylated uroplakins stimulates numerous host signaling pathways as the host tries to clear the bacteria from the bladder (Mysorekar et al., 2002). Normally inert, the bladder epithelium immediately activates genetic programs upon binding. These pathways trigger apoptosis of the superficial epithelial layer resulting in their exfoliation into the bladder lumen, where the cells and bound bacteria may be eliminated with the flow of urine (Mulvey et al., 1998). Many other genetic programs are also activated, promoting growth of the underlying transitional epithelial cells to regenerate the superficial layer (Mysorekar et al., 2002). Moreover, bacterial binding by means of type 1 pili stimulates innate host immune defenses by facilitating delivery of bacterial lipopolysaccharide (LPS) to Toll-like receptor 4 (TLR4) present on the host epithelial cell surface (Schilling et al., 2001, 2003a,b). This stimulation results in expression and release of inflammatory cytokines, such as IL-6, which recruit numerous neutrophils into the bladder in an effort to clear the infection (Haraoka et al., 1999; Schilling et al., 2001).

UPEC subverts these defenses by invading into the superficial epithelial cells. FimH also mediates this invasion and signals uptake of the bacteria via a zipper-like mechanism involving recruitment of host phosphoinositide 3-kinase, focal adhesion kinase, α-actinin, and vinculin (Martinez et al., 2000). This signaling cascade leads to host actin rearrangements that engulf and internalize the bacteria. Bacterial invasion results in an intracellular, protective niche for the bacteria, which replicate in the cytoplasm to form disorganized intracellular clusters (Justice et al., 2004; Mulvey et al., 2001). The bacteria then undergo a morphological change whereby they shorten from their typical rod shape into a much more coccoid morphology (Justice et al., in preparation). Concurrent with this morphological shift is a compaction of the intracellular clusters into a tight-knit biofilm-like community (Anderson et al., 2003; Justice et al., in preparation). Continued replication results in a bulge on the surface of the bladder epithelium with the appearance of a pod (Anderson et al., 2003) (Figure 10A, B). Each pod consists of an individual superficial bladder cell completely filled up with the intracellular biofilm (Anderson et al., 2003). The bladder epithelium becomes coated with pods, while individual bacteria are rarely seen on the bladder surface at this stage (Figure 10C). Each pod features bacterial subpopulations differentially expressing type 1 pili, antigen 43 (an autotransporter adhesin), and possibly other factors, and the bacteria are encased in a polysaccharide matrix (Anderson et al., 2003). Each bacterium in the pod is coated with fibers, the tips of which are buried in the matrix, seemingly providing spatial

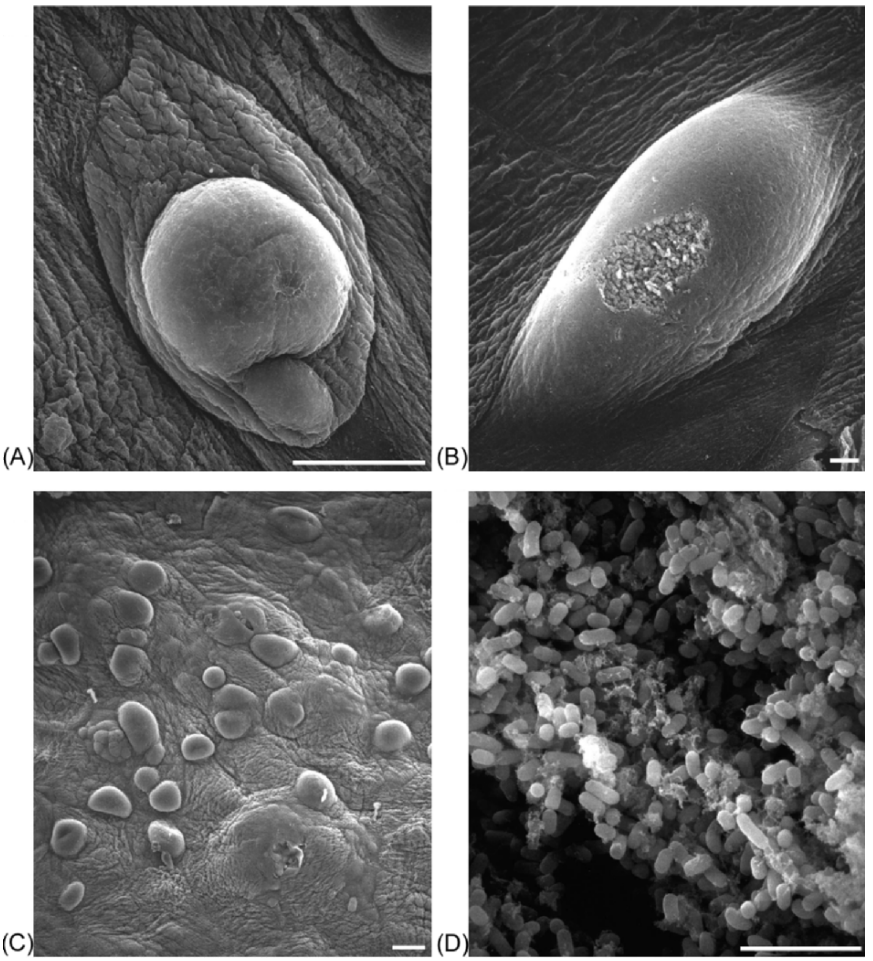

FIGURE 10. Pod formation induced by UPEC in the superficial cells of the bladder. Scanning electron micrographs show that UPEC form biofilms inside the superficial cells of the bladder, which extend into the bladder lumen and appear as pod on the bladder surface. (**A** and **B**) Individual superficial cells become filled with bacteria and bulge out from the epithelial surface. Bacteria are protected inside the pod underneath the host cell membrane and coated with a shell of uroplakin. Scale bars, 50 μm (A), 5 μm (B). (C) The bladder surface becomes inundated with pods. This appears to be a mechanism used by UPEC to quickly increase their titers in the bladder and may impact transmission and persistence of the organism in the urinary tract. Scale bar, 50 μm. (D) Numerous fibers apparently connect the bacteria and provide support for individual bacteria and the biofilm as a whole. Scale bar, 5 μm.

support for the bacteria in the biofilm (Anderson et al., 2003) (Figure 10D). These fibers might represent glycocalyx expressed by the bacteria, contributing to the polysaccharide matrix, or perhaps they are novel adhesive pili, binding to receptors in an effort to maintain the pod architecture. Pods are only seen in the superficial cells of the bladder, and these cells remain attached to the epithelium despite exfoliation of the surrounding epithelium.

Bacteria do not remain in this intracellular biofilm indefinitely, but instead detach and flux out of the superficial epithelial cells. Bacteria on the edge of the pod begin to differentiate into highly motile rods that swim out of one end of the cell (Justice et al., in preparation). Eventually, the pod ruptures, spilling hundreds of thousands of individual bacteria into the lumen (Mulvey et al., 2001). Often, these fluxing bacteria are filamentous, which can protect UPEC from immune clearance and may facilitate binding of neighboring cells (Justice et al., in preparation; Mulvey et al., 2001). Indeed, rebinding of the epithelium, possibly by means of FimH on type 1 pili, leads to a second round of pod formation, albeit at a slower rate (Justice et al., in preparation). Ultimately, subsequent rounds of pod formation and fluxing discontinue, and UPEC forms a quiescent reservoir of intracellular clusters of 2–4 bacteria each (Justice et al., in preparation; Mulvey et al., 2001). This reservoir lasts for months despite antibiotic treatments (Mulvey et al., 2001; Schilling et al., 2002). Activation of this reservoir seemingly provides the seed for recurrent infections, and the activated bacteria most likely express type 1 pili to bind to the superficial epithelium and reinitiate the pathogenic cycle (Mulvey et al., 2001; Schilling et al., 2002). In this way, specific adhesins allow UPEC to create a highly tenacious niche wherein the bacteria can rapidly form high titers in the bladder. Fluxing from pods facilitates rebinding of the bladder and establishment of a persistent reservoir, and also probably impacts community transmission as numerous pathogens are suddenly reintroduced into the bladder lumen and excreted into the environment with the flow of urine. In recent years, widespread clonal distribution of multidrug-resistant UPEC strains causing UTI have underscored the need for development of new therapies to deal with these emerging trends (Manges et al., 2001; Mazzulli, 2002). Considering the numerous steps of the UPEC pathogenic cycle involving adhesins, and the many novel adhesins emerging through genomic analyses, therapeutics targeting adhesins and adhesive structures may very well prove quite effective at abrogating pathogenesis and eliminating UTI.

4. Adhesin-based Technology

Due to the rise of antibiotic-resistant bacteria, more (and better) therapeutics must be developed in order to fight or prevent infections caused by these bacteria. Because of the vital role of adhesins in the pathogenesis of numerous bacterial pathogens, it seems likely that technologies targeting adhesins would be effective in inhibiting bacterial infections. Some approaches to accomplish

this goal involve development of vaccines directed against adhesive structures, while other tactics entail administration of receptor analogues. In either case, the underlying theory is the disruption of binding events between adhesins and receptors to prevent bacterial attachment to host tissues.

4.1. Vaccine Strategies

The goal of vaccination against adhesive structures is prevention of bacterial binding and colonization of host tissues. Central to this vaccine strategy is the conservation of adhesin sequence across many pathogenic strains of the same organism (Wizemann et al., 1999). For the bacterium, this invariance is necessary to maintain the ability to recognize its specific receptor. However, adhesin conservation is advantageous for vaccination by permitting immune recognition of a panel of pathogenic strains after vaccination by a single clone. Specific recognition of adhesins by antibodies assists opsonization of the bacterium and also blocks adherence by tying up the adhesins and making them unavailable for binding to host sites (Wizemann et al., 1999). For instance, vaccination with FimH might inhibit the ability of UPEC to bind and invade into the bladder epithelium, and if left in the bladder lumen, these organisms could more easily be attacked by immune defenses or be eliminated from the host by the flow of urine. Such FimH vaccination in animal models have shown great success in preventing bladder colonization and in reducing the bacterial load in the bladder (Langermann and Ballou, 2001; Langermann et al., 1997; Wizemann et al., 1999). In addition to blocking initial binding, an adhesin-based vaccine might be effective in disruption of the pod architecture or in blocking subsequent binding events after fluxing from pods. Such a vaccine may even lead to prevention of UTI recurrences. Thus, vaccination against adhesins can potentially inhibit multiple steps during bacterial pathogenesis. Another immunization strategy is the administration of multivalent vaccines, which induce an immune response against several different molecules from the same microorganism or from different microorganisms. The multivalent DTaP vaccine has been used, with great success, to immunize against diphtheria, tetanus, and pertussis, caused by *Corynebacterium diphtheriae*, *Clostridium tetani*, and *B. pertussis*, respectively (Feldman, 2001). The components of this vaccine include toxins from each of these pathogens as well as several adhesins of *B. pertussis* (Feldman, 2001; Watanabe et al., 2002). Depending upon the manufacturer, DTaP may include FHA, pertactin (an autotransporter adhesin), or subunits of adhesive chaperone/usher pili (Feldman, 2001; Watanabe et al., 2002). This vaccine has been quite effective at reducing the incidence of pertussis, in large part due to the production of blocking antibodies and the inhibition of bacterial binding to host tissues.

As a twist on vaccination against adhesins, there have been many studies demonstrating heterologous display of immunogens on adhesive structures such as pili (Klemm and Schembri, 2000; Lee et al., 2003). This technique covalently fuses epitopes as inserts in pilus subunits, which, when assembled into

pili, could be used for immunization or other applications that might benefit from surface display of peptides (Klemm and Schembri, 2000; Lee et al., 2003). In this way, peptides that normally would be in low abundance or partially inaccessible to the immune system can be surface-expressed on thousands of pili with thousands of subunits each (Klemm and Schembri, 2000). For instance, in one study, the cholera toxin B (CTB) subunit was fused to FimA, which, when used for immunization, conferred immune recognition of CTB (Klemm and Schembri, 2000; Lee et al., 2003). Such peptide display strategies demonstrate the versatility and many potential uses of adhesins, and this technology may very well lead to better or cheaper vaccine development.

4.2. Receptor Analogues

Another promising area of research involves the development of receptor analogues, soluble molecules that mimic receptor structures and tie up adhesins so that they are prohibited from adhering to their natural receptors (Kelly and Younson, 2000). This antimicrobial strategy might be effective in preventing infections for which suitable vaccines or antibiotics are unavailable (Karlsson, 1998). Receptor analogues have been shown to be effective at inhibiting colonization of a variety of bacterial pathogens, including *Streptococcus pneumoniae*, *P. aeruginosa*, and *Helicobacter pylori*, as well as many different viral pathogens (Karlsson, 1998; Kelly and Younson, 2000). Because many adhesins recognize glycoprotein receptors, soluble carbohydrate molecules have been used extensively to inhibit binding interactions (Karlsson, 1998; Kelly and Younson, 2000). It has been suggested for years that cranberry juice can inhibit or even prevent recurrent UTIs and infection with UPEC (Kontiokari et al., 2001; Krieger, 2002; Reid, 1999). Binding of type 1 pili and P pili can be inhibited by fructose and proanthrocyanidins found in cranberry extracts, potentially by acting as receptor analogues, and some clinical trials have shown reductions in UTI rates among those who drink cranberry juice (Kontiokari et al., 2001; Reid, 1999). The commercial availability of cranberry juice would allow self-medication and might reduce visits to doctors' offices. However, some researchers have found no effect of cranberry consumption on UTI occurrence (Krieger, 2002). Thus, the use of cranberry juice remains controversial. Clearly, more research is needed to understand the role, if any, of cranberry juice in preventing UTIs. Still, it is intriguing to consider the potential uses of receptor analogues in disrupting adhesin–receptor interactions and thwarting microbial infections.

5. Conclusions

One of the very early steps in pathogenesis of many bacteria is binding of host tissues. Adhesins target bacteria to a specific host site, the interactive surfaces of the adhesin recognizing defined receptor structures at these sites.

Because there are many different host niches that may be colonized, there are a multitude of different adhesins produced that allow various microorganisms to bind. The different adhesive strategies used by bacteria exemplify the extreme diversity of these microbes and suggest mechanisms of immune evasion. At the same time, striking similarities and common themes exist between related bacterial adhesive structures, and even with structurally dissimilar adhesins. These similarities probably are the result of convergent evolution, as highly divergent bacteria cope with the common task of binding. Conserved sequences of adhesive pili generally appear to be involved in interactions between pilus subunits, while variant residues face the environment, and hence the immune system. This allows the bacteria to vary their adhesive pili but retain the ability to efficiently assemble the adhesin. The conserved adhesin subunit of pili is but one small part of a gigantic macromolecular structure and may not be as accessible to immune detection as the rest of the pilus rod. Afimbrial structures might avoid immune recognition because of the comparatively small amount of protein exposed on the bacterial surface. Also, it is possible that cleavage and release of autotransporters may permit the bacteria to shed these antigens to prevent antibody detection. The ability to form biofilms and/or invade into deeper tissues further permits escape from host defenses and allows the microbes to reach new sites. At these sites, other adhesins may come into play in an effort to establish itself in a new niche. Underlying all of these processes is the activation of signaling and regulatory pathways in the bacterium, which alerts the microorganism of changes in the surrounding environment and orchestrates proper timing of bacterial virulence. Manipulation of the host is a vital part of many infectious processes, which, in many cases, is also carried out by the adhesin. The multifunctionality of adhesive structures bestows upon bacteria the ability to carry out several different pathogenic steps with the production of a single factor. Disruption of these interactions would have serious consequences for the infecting microorganism, and thus more research is needed to understand the mechanisms of adhesin binding, recognition of receptors, downstream signaling pathways in the bacterium and the host, and the consequences of binding for individual pathogens. With greater understanding of these processes, better therapeutics may be developed, whether with antibiotics, vaccines, or receptor analogues. In any event, it is imperative to understand adhesins and attachment to the host, the crucial first step in pathogenesis, without which, the infection would not exist.

Questions to Consider

1. What advantage might a bacterium gain by producing more than one adhesive structure?

Many adhesive structures on bacteria are highly specific for particular receptors. This allows the bacteria to bind tightly to tissues or other surfaces and

to establish advantageous niches in their environment. Through the use of more than one adhesin, a bacterium can potentially colonize multiple surfaces with high specificity. This ability may then aid in the survival of the bacterium by allowing them to survive, even when transmitted to different surroundings.

2. Explain the process of donor strand complementation and donor strand exchange that occurs in type 1 and P pili.

Pilin domains that make up the superstructure of the pilus are composed of immunoglobulin folds, except that the seventh β-strand is missing. The chaperone donates a strand to complete the fold and stabilize the pilin subunit. Donor strand exchange takes place at the usher. The chaperone primes the pilin subunit for accepting the N-terminal extension of the next pilin subunit to be assembled into the pilus, as a donor strand inserts in an antiparallel β-strand pairing.

3. Compare and contrast type 1 pilus structure and P pilus structure.

Type 1 pili and P pili are assembled by the chaperone/usher pathway of pilus assembly. Both form rigid, helical rods that extend from the bacterial outer membrane. A thinner tip fibrillum, comprising adaptor subunits, is attached to the distal end of both pili, and a lectin-binding adhesin sits at the tip.

However, several differences exist between type 1 pili and P pili. First, the globoside binding site of PapG resides on the side of the lectin domain, instead of the tip as in FimH. Also, P pili contain a longer tip fibrillum, comprising repeated monomers of PapE. This longer fibrillum increases flexibility of the pilus tip, and allows for the proper orientation of the adhesin during binding.

4. Describe the strategies that Gram-positive bacteria may use to present adhesive organelles on their surfaces. Why are these methods different from those used by Gram-negative bacteria?

The cell envelope of Gram-negative bacteria consists of an inner membrane, periplasmic space, and an outer membrane. In contrast, Gram-positive bacteria lack an outer membrane and instead possess a very thick peptidoglycan layer. Thus, these organisms must utilize different strategies to secrete adhesive organelles onto the cell surface. One way that Gram-positive bacteria display adhesive structures is through covalent linkage to the peptidoglycan. Other methods used to anchor adhesive organelles include noncovalent interactions with teichoic acids, insertion of stretches of hydrophobic amino acid residues into the membrane, and anchoring at the membrane through lipoproteins.

5. What proteins interact with the Tir protein from enteropathogenic *E. coli* to form a complex to initiate actin polymerization when it is phosphorylated?

Nck, N-WASP, and Arp2/3.

6. What adhesins are involved in attachment of *Yersinia* to M cells.

The pH6 antigen, Ail protein, and YadA protein.

7. What characteristics of adhesins and adhesive interactions make them attractive targets for vaccine design?

Bacterial binding to host tissues typically involves a precise interaction between a specific adhesin and a defined receptor molecule. In order for the bacterium to retain the ability to recognize its host receptor, the adhesin sequence and structure must be conserved across many pathogenic strains of the same organism. Vaccines can take advantage of this invariance by inducing immune recognition of potentially all strains of an organism that express the conserved adhesin.

Because adhesion is often the first step in bacterial pathogenesis, vaccination against adhesins can abrogate infection at its initial stages. Further, adhesive contacts play a major role in biofilms and other host–pathogen interactions. Thus, adhesin-based vaccines may disrupt pathogenesis at several points during an infection.

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Chapter 8

Bacterial Invasion into Non-Phagocytic Cells

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Historical Landmarks

1964 Development of tissue culture model for studying bacillary dysentery (LaBrec et al., 1964).

1967 Demonstration of *Salmonella* penetration into epithelial cells (Takeuchi, 1967).

1970 Demonstration of *Listeria monocytogenes* penetration into epithelial cells (Racz et al., 1970).

1973 Development of tissue culture model for studying *Salmonella* invasion (Giannella et al., 1973).

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1987 Identification of *Yersinia pseudotuberculosis* invasin (Isberg et al., 1987).

1987 Observation of *Shigella*-induced cytoskeleton rearrangements (Clerc and Sansonetti, 1987; LaBrec et al., 1964).

1989 Identification of *Salmonella* invasion genes (Galán and Curtiss, 1989).

1990 Identification of β1 chain integrins as the receptors for *Y. pseudotuberculosis* invasion (Isberg and Leong, 1990).

1991 Identification of *Listeria* invasion determinant: internalin (Gaillard et al., 1991).

1992 Observation of *Salmonella*-induced cytoskeleton rearrangements upon bacterial entry (Francis et al., 1992, 1993).

1992 Demonstration of *Shigella flexneri* entry into human colonic Caco-2 epithelial cells occurs through the basolateral side (Mounier et al., 1992).

1996 Identification of human E-cadherin as the cellular receptor for *L. internalin* (Mengaud et al., 1996).

1998 Identification of SopE as exchange factors for small molecular weight GTP-binding proteins of the Rho subfamily (Hardt et al., 1998a).

1999 Discovery of first bacterial encoded actin-binding protein in *Salmonella* (Zhou et al., 1999b).

1. Introduction

It is well accepted that mammals and bacteria coexist without adverse effects upon the host under normal circumstances. Both physical and immunological host barriers keep the bacteria from entering the internal organs and bloodstream. Bacterial infection through skin is rare unless there is injury that compromises the integrity of the intact skin layer. Similarly, the intestinal epithelia serve as an effective barrier to prevent numerous intestinal microorganisms from entering into deeper tissues while permitting the intake of nutrients into the bloodstream. It is known that the intestinal epithelia absorb nutrients through pinocytosis and receptor-mediated endocytosis, but they prevent the uptake of large particles, such as bacteria. Thus, most intestinal intracellular pathogens have evolved mechanisms to induce their own uptake into the intestinal epithelial cells.

Intracellular pathogens are the causative agents for a number of diseases ranging from mild local inflammations to more severe systemic infections. Despite intensive research efforts, illness due to these pathogens continues to be a major public health issue for both humans and animals. In addition, many multidrug-resistant strains are emerging and becoming a worldwide problem. In general, there are two different ways bacterial pathogens invade host cells. One way is to enter host professional phagocytes. The other way is

to actively induce host cell cytoskeleton rearrangements to induce uptake in nonphagocytic cells, such as the intestinal epithelium. Due to recent developments and advances in molecular genetics and cell biology, significant progress has been made in our knowledge of the invasive mechanisms of obligate and facultative intracellular pathogens including *Listeria*, *Salmonella*, *Shigella*, and *Yersinia* spp. As a result, the precise molecular mechanism underlying their pathogenesis is beginning to come to light.

All invasion processes involve the modulation of host signaling pathways and the disruption of the host cell cytoskeleton at certain stages during entry of the pathogen. Different bacterial pathogens have evolved distinct strategies to engage the host machinery to promote their entry. While some use bacterial surface components to bind host cell surface receptors to trigger the signal transduction pathways (Cossart and Lecuit, 1998; Isberg and Barnes, 2001), others send bacterial effectors directly inside the host cells to modulate the signaling components and cytoskeletal machinery (Galán and Zhou, 2000). Two classical examples representing these two types of bacterial entry mechanisms are the "trigger" mechanism represented by *Salmonella* and *Shigella* entry, and the "zipper" mechanism illustrated by *Listeria* and *Yersinia* invasion (Figure 1). The "zipper" mechanism involves the engagement of a bacterial ligand with a host cell receptor leading to the gradual movement of the plasma membrane alongside the surface of the invading bacteria which eventually wraps the bacteria inside the host cell membrane. This entry process shares many similar mechanisms as the typical C3 receptor-mediated phagocytosis by professional phagocytes (Kaplan, 1977). In contrast, the

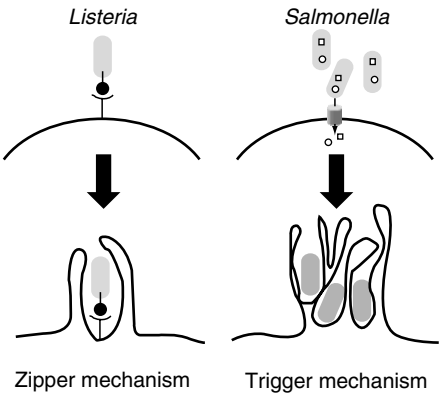

FIGURE 1. The "zipper" and "trigger" bacterial entry mechanisms. The "zipper" mechanism involves the engagement of a bacterial ligand with a host cell receptor leading to the gradual movement of the plasma membrane alongside the surface of the invading bacteria and eventually wraps the bacteria inside the host cell membrane. The "trigger" mechanism induces dramatic actin cytoskeleton rearrangements and membrane ruffling leading to macropinocytosis and bacteria entry.

"trigger" mechanism induces dramatic actin cytoskeleton rearrangements and membrane ruffling leading to macropinocytosis and bacteria entry. While the "trigger" mechanism often involves multiple bacterial effectors, one bacterial factor is usually required and sufficient for inducing bacteria entry by the "zipper" mechanism. This chapter will discuss the molecular basis of the two entry mechanisms using *Salmonella enterica* serovar Typhimurium and *Listeria monocytogenes* as two model organisms.

Both salmonellosis and listeriosis are common food-borne pathogens that initiate their infection through the host intestinal tract. *S. enterica* serovar spp. are a group of Gram-negative bacteria that are usually motile and are pathogenic for humans and other warm-blooded animals. *Salmonella* strains cause food poisoning, gastrointestinal inflammation, typhoid fever, and septicemia in humans. The most common source of human infection is through ingestion of contaminated food. The virulence determinants needed for *S. enterica* serovar Typhimurium are similar to those of other intestinal pathogens: (1) it must successfully survive the hostile acidic environment in the stomach before colonizing the small intestine; (2) once in the intestine, the bacteria must breach the barrier of intestinal epithelial cells; and (3) the bacteria must survive inside host cells including professional macrophages. Pathogenic *Salmonella* spp. have evolved complex systems that enable the organism to respond and survive the low pH in the stomach and to reach M cells and enterocytes in the small intestine. An essential virulence feature in *Salmonella* pathogenesis is the ability to enter nonphagocytic eukaryotic cells and to exist as intracellular parasites inside enclosed vacuoles (Moulder, 1985; Takeuchi, 1967). The intracellular environment provides a unique niche for the bacteria to multiply, evade host immune responses, and spread systemically. Entry into nonphagocytic epithelial cells involves a series of complex interactions between *Salmonella* and the host cell. Upon contact with intestinal epithelial cells, *Salmonella* inject a set of bacterial proteins (effectors) into host cells via the bacterial type III secretion system (T3SS) (see Chapter 9). Some of these effectors activate the host cell signal transduction pathways to initiate actin cytoskeleton rearrangements. Other effectors, namely the actin-binding proteins, directly modulate host cell actin dynamics to facilitate bacterial uptake.

Unlike *S. enterica* serovar spp., *L. monocytogenes* is a Gram-positive food-borne pathogen that causes localized gastroenteritis and more serious illness by disseminating from the intestinal lumen to the central nervous system and the fetoplacental unit. After surviving the hostile environment in the stomach, the bacteria invade the intestinal epithelium to initiate the disease process. It is well accepted that one essential virulence feature of *L. monocytogenes* is their ability to breach the intestinal, placental, and blood–brain barriers. Two *L. monocytogenes* surface proteins, internalins InlA and InlB, have been identified to be necessary and sufficient for mediating invasion through receptor-mediated endocytosis into a variety of cell types.

Shigella and *Yersinia* spp are two other pathogens that are well known to enter nonphagocytic mammalian cells utilizing the "trigger" and "zipper"

mechanisms, respectively. *Shigella flexneri* is a Gram-negative bacillus that causes bacillary dysentery and is very closely related to *Escherichia coli. Shigella* is able to invade nonphagocytic epithelial cells via bacteria-induced actin cytoskeleton rearrangements similar to those induced by *Salmonella,* although the molecular mechanism is less well understood. Despite the remarkably similar morphological membrane projection during entry, there are a number of significant differences. First, *Shigella* can invade polarized cells only from their basolateral side (Mounier et al., 1992), while *Salmonella* is capable of entering through both the apical and the basolateral sides. Second, while *Salmonella* remains in a vacuole after entry, *Shigella* escapes into the cytoplasm following entry. Third, *Shigella* moves intracellularly and intercellularly to spread from cell to cell (Vasselon et al., 1991, 1992). *Yersinia pseudotuberculosis* and *Y. enterocolitica* enter nonphagocytic cells via the "zipper" mechanism. The invasin, a bacterial surface protein, mediates both attachment and bacterial entry (Isberg et al., 1987; Young et al., 1990). Remarkably, noninvasive *E. coli* expressing *Yersinia* invasin or latex beads coated with recombinant invasin render them capable of entering epithelial cells (Isberg et al., 1987; Young et al., 1992). Binding of invasin to β1 integrin receptor results in the activation of a signal cascade leading to actin cytoskeletal rearrangements and bacterial entry, which resembles the classical receptor-mediated endocytosis (Grassl et al., 2003; Gustavsson et al., 2002; Wiedemann et al., 2001).

2. *Salmonella* Invasion

Salmonella entry into nonphagocytic cells is a classical example of the "trigger" mechanism. *S. enterica* serovar Typhimurium has evolved the ability to coordinately deliver a panel of bacterial proteins into host cells to induce bacterial uptake by modulating the host actin cytoskeleton dynamics both directly and indirectly. Indirectly, these bacterial proteins subvert host cell signal transduction pathways to induce actin cytoskeletal rearrangements and membrane ruffling. Recent studies have indicated that ruffle formation is also regulated directly by bacterial encoded actin-binding proteins that mimic the functions of the host cellular actin-binding proteins (Hayward and Koronakis, 1999; Zhou et al., 1999b). The concerted actions of these bacterial effector proteins eventually lead to the efficient uptake of the bacterium (Galán and Zhou, 2000). Thus, the ability of *Salmonella* effectors to coordinately regulate host actin cytoskeleton rearrangements is the result of functional mimicry of host functions by the type III translocated effectors (see Chapter 9).

2.1. Pathogenicity Islands and Type III Protein Secretion Systems

Several studies have led to the identification of genes that are required for *Salmonella* pathogenesis, in particular for *Salmonella* invasion into

nonphagocytic cells (Blanc-Potard and Groisman, 1997; Galán and Curtiss, 1989; Ochman et al., 1996; Shea et al., 1996; Wong et al., 1998; Wood et al., 1998). Many of these virulence genes and operons are located in large genetic elements of the *Salmonella* chromosome. Since these large elements are absent from the chromosome of closely related *E. coli*, they are termed pathogenicity islands (see Chapter 4). Virulence plasmids also contribute to *Salmonella* virulence and survival in macrophages (Guiney et al., 1994; Gulig, 1990; Wallis et al., 1995). At least five pathogenicity islands have been identified in *Salmonella* (Blanc-Potard and Groisman, 1997; Galán and Curtiss, 1989; Ochman et al., 1996; Shea et al., 1996; Wong et al., 1998; Wood et al., 1998) that contribute to virulence at defined stages of the infection process.

Salmonella Pathogenicity Island I (SPI-1) is the best-studied pathogenicity island. It is located at centisome 63 on the *Salmonella* chromosome and is approximately 43 kb in length. A number of studies have demonstrated that SPI-1 is required for *Salmonella* entry into epithelial cells (Galán and Curtiss, 1989) lining the surface of the intestine. This is consistent with the fact that SPI-1 mutants are defective in virulence when administered orally, but not if given systemically (Galán and Curtiss, 1989). Mutants that are defective in entry into epithelial cells were also found to be avirulent in studies using the mouse-typhoid model (Galán and Curtiss, 1989) and in calves when administered orally (Tsolis et al., 1999a, 2000; Watson et al., 1998).

SPI-2, SPI-3, and SPI-4 are situated at centisome 31, 82, and 92 of the *Salmonella* chromosome. Genes in these three islands are essential for *Salmonella* survival and growth in the host (Hensel et al., 1997, 1998; Shea et al., 1999; Vazquez-Torres et al., 2000). SPI-5 was originally found to be involved in inflammation and fluid secretion in the intestine (Galyov et al., 1997; Norris et al., 1998; Wood et al., 1998). It was recently shown that at least one gene in this island (*sopB*) is also involved in the *Salmonella* invasion process (Galyov et al., 1997; Galán and Zhou, 2000; Hong and Miller, 1998; Terebiznik et al., 2002).

SPI-1 and SPI-2 encode specialized protein secretion and translocation systems termed T3SS. The other three islands encode no such secretion systems but rather appear to encode secreted effector proteins that feed through the T3SS. Genes in SPI-1 can be divided into three groups: (1) genes that encode the actual secretion/translocation apparatus; (2) genes that encode proteins that are secreted and/or translocated into host cells; and (3) genes that encode proteins involved in gene regulation of the T3SS.

Recently, the SPI-1 secretion apparatus was shown by electron microscopy to constitute a "needle complex" that is similar to the bacterial flagella system both biochemically and structurally (Kubori et al., 1998). Purified needle complexes consist of at least three proteins encoded in SPI-1 (PrgK, PrgH, and InvG). Mutations in *prgK*, *prgH*, and *invG* have been shown to abolish the secretion of a panel of *S. enterica* serovar Typhimurium proteins (e.g., SipA, SipB, and SipC). The subsequent translocation of these bacterial proteins into eukaryotic host cells is required for *Salmonella*

invasion into nonphagocytic epithelial cells. Secretion has been reported to require host cell contact (Zierler and Galán, 1995). However, these proteins are also secreted under certain laboratory conditions in sufficient amounts to facilitate their studies in the absence of host cells. These secreted proteins can be visualized by SDS-PAGE from supernatants of *S. enterica* serovar Typhimurium cultures under such inducing conditions. At least 13 proteins that are delivered by the SPI-1 T3SS have been identified: AvrA, SipA, SipB, SipC, SipD, SlrP, SopA, SopB, SopD, SopE, SopE2, SptP, and SspH1 (Bakshi et al., 2000; Hardt and Galán, 1997; Hardt et al., 1998a, b; Jones et al., 1998; Kaniga et al., 1995a, b, 1996; Miao et al., 1999; Stender et al., 2000; Tsolis et al., 1999b; Wood et al., 1996, 2000). During the infection process, these proteins are thought to be translocated into the host cell, where they engage host cell machinery to promote bacterial uptake and subsequent survival (Galán, 1998, 1999). Although previous studies have shown that SPI-1 is required for *Salmonella* invasion of epithelial cells, the molecular mechanism has been only recently elucidated. The hallmark of *Salmonella* entry into host cells is the profuse host actin cytoskeletal rearrangements at the site of *Salmonella* contact with intestinal epithelial cells (Francis et al., 1993; Galán and Zhou, 2000; Ginocchio et al., 1994) (Figure 2). These massive actin cytoskeleton rearrangements and subsequent entry of *Salmonella* are completely abolished when actin polymerization is inhibited by cytochalasins (Finlay et al., 1991). Effectors of the type III secretion/translocation system of SPI-1 are required for inducing such actin cytoskeleton rearrangements (Galán and Curtiss, 1989).

2.2. *Actin Cytoskeleton Rearrangements and* Salmonella *Entry into Host Cells*

To understand the molecular mechanisms of how *Salmonella* effectors modulate the host actin cytoskeleton, it is necessary to briefly discuss the components of the actin cytoskeleton and how their functions are affected by various regulators in mammalian cells. The actin cytoskeleton is responsible for cell movement, cytokinesis, and the organization of organelles within the cell. Three types of cytoskeleton components exist to provide both movement and stability in the mammalian cell: microfilaments (MFs), microtubules (MTs), and intermediate filaments (IFs). Each of these cytoskeleton structures is dynamic in nature and composed of polymers of subunits. MFs are assembled from monomers consisting of actin, MTs from tubulin, and IFs from IF proteins. In nonphagocytic epithelial cells, MFs project into the villi, giving shape to the cell surface. MTs grow out of the centrosome to the cell periphery. IFs connect adjacent cells through desmosomes.

MFs are the most elastic among the three types of cytoskeleton components. The actin cytoskeleton plays vital roles in many cellular processes, including cell movement and endocytosis (Barkalow and Hartwig, 1995;

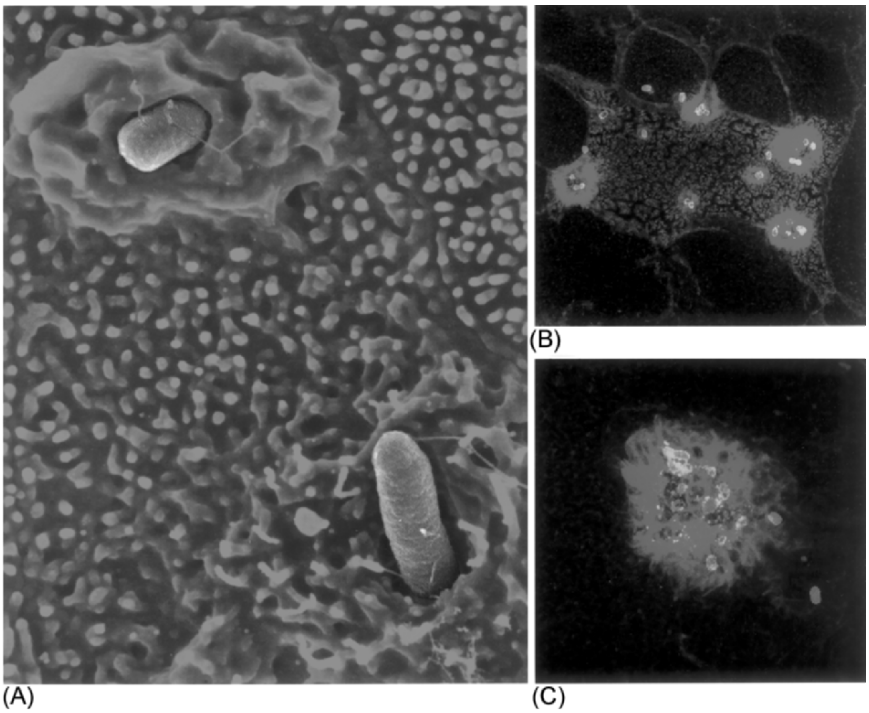

FIGURE 2. Interaction of *Salmonella typhimurium* with intestinal epithelial cells. (Adapted with permission from Zhou and Galán, 2001.) (A) Scanning electron micrograph of *S. typhimurium*-infected intestinal epithelial Caco-2 cells. (B) and (C) Actin cytoskeleton rearrangements in *S. typhimurium*-infected intestinal epithelial Caco-2 cells. Filamentous actin was stained with rhodamine phalloidin and *S. typhimurium* with a FITC-conjugated antibody.

Gottlieb et al., 1993; Hartwig et al., 1985; Lamaze et al., 1997). Based on their architectural differences, there are three major classes of actin cytoskeleton structures in cells: stress fibers, lamellipodia, and filopodia (Small et al., 1999). Stress fibers, which are primarily bundles of actin, are distributed at the bases of cultured cells. Lamellipodia and filopodia are both cellular actin extensions involved in cellular movement. Lamellipodia differ from filopodia in that they are defined by a more sheet-like actin meshwork rather than microspike-like actin bundles found in filopodia.

The actin cytoskeleton is highly dynamic and tightly regulated in cells. Actin can exist in a monomeric globular form termed G-actin, or in highly ordered multimers termed filamentous actin, or F-actin. Intracellular levels of G-actin and F-actin are maintained at a dynamic steady state by a number of actin-binding proteins. In addition, the concentration of free G-actin in the cell is kept below the level (critical concentration) that is required for

actin polymerization. Although net levels of G-actin and F-actin may be kept relatively constant, actin molecules are constantly exchanging between G-actin and F-actin. F-actin depolymerizes from one end of the filaments and polymerizes at the other end while the net length can be kept constant, a process called treadmilling. This dynamic nature ensures that the cell can respond to environmental cues in a timely fashion while maintaining the integrity of the actin cytoskeleton architecture.

Actin dynamics are regulated by a number of factors including pH, ionic strength, phosphoinositides, Rho family GTPases Cdc42 and Rac, and a number of actin-associated proteins. The low–molecular weight Rho family GTPases have a critical role in the dynamic regulation of the actin cytoskeleton. These GTPases function as molecular switches by binding to GDP in the basal or nonactivated state and GTP in the activated state. The binding of GTP results in a structure conformational change allowing the protein to bind to downstream effectors. As their names suggest, all have an intrinsic GTPase activity. The hydrolysis of GTP results in converting the protein to the basal, GDP-bound state shifting it from active to nonactive state. Likewise, GDP can be exchanged by GTP and result in the activation of these GTPases. As the intrinsic rate of hydrolysis and nucleotide exchange is slow, the hydrolysis of GTP to GDP is greatly stimulated by GTPase-activating proteins (GAPs), and the exchange is facilitated by guanine nucleotide exchange factors (GEFs). All known GEFs specific for the Rho family GTPases possess the Dbl homology (DH) domain. This domain binds the switch I and II region, which is responsible for the conformational change between inactive and active states of the Rho GTPases.

Many actin-binding proteins have been identified that play different, yet coordinated, roles in regulating the actin cytoskeleton. These proteins include: G-actin-binding/sequestering proteins, such as profilin (Theriot and Mitchison, 1993); F-actin-capping proteins, such as gelsolin (Sun et al., 1999); F-actin-severing proteins, such as ADF/cofilin (Bamburg, 1999), and F-actin-bundling proteins, such as plastin (fimbrin) (Bartles, 2000). Among these actin-binding proteins, only ADF/cofilin and plastin have been studied in detail for their roles in bacterial entry (Adam et al., 1995; Bierne et al., 2001; Zhou et al., 1999a). ADF/cofilin are a group of actin-binding proteins that modulate actin dynamics by promoting depolymerization of actin filaments (Bamburg, 1999; Carlier et al., 1997; Southwick, 2000; Theriot, 1997). The activities of ADF/cofilin are inhibited by phosphorylation at the third Serine residue located at the N-terminus (Agnew et al., 1995; Moriyama et al., 1996) and by PtdIns (4,5)P_2 binding (Yonezawa et al., 1990). Actin-binding proteins are often found to be multifunctional, especially when assayed under different experimental conditions in vitro. The precise in vivo function of actin-modulating proteins is likely to depend on both their concentration and spatial localization. Actin nucleation and polymerization are also tightly regulated by a number of actin-binding proteins and signal transduction molecules such as the Arp2/3 complex and Cdc42 (Blanchoin et al.,

2000; Tapon and Hall, 1997; Yarar et al., 1999). Not surprisingly, many pathogens including *Salmonella* strains have evolved to intercept and redirect these regulatory steps to reorganize the actin cytoskeleton machinery for their own advantages. At the same time, the complex regulation also demands that any intervention by the bacteria must be precisely controlled to ensure the coexistence and viability of both the bacteria and the host cells.

A model for *Salmonella* invasion into host cells has emerged based on recent advances in the understanding contributions from individual *Salmonella* effectors (Figure 3). Upon contact with the host cells, *Salmonella* deliver a panel of effector proteins into the host cells. Delivery of the exchange factor SopE and the inositol polyphosphatase SopB results in the activation of Cdc42 and Rac-1, the stimulation of downstream signaling pathways, and the recruitment of Arp2/3 complex, plastin, and other ruffling-associated molecules. This leads to the initiation of the actin cytoskeleton reorganization. SipA and SipC participate in this process by nucleating the actin assembly, lowering the critical concentration of actin, and stimulating the bundling activity of plastin. SopB also helps the membrane fission process by decreasing the local concentration of PIP_2 at the base of the membrane ruffles. The combined activities of these effectors result in a localized and pronounced outward extension of actin filaments and the membrane ruffles resulting in the engulfment of *Salmonella* in an enclosed membrane compartment. In addition, immediately following the translocation of SPI-1 type III effectors, slingshot-mediated dephosphorylation leads to the brief activation of ADF/Cofilin. This results in increased treadmilling of actin filaments at the site of bacterial entry, leading to outward protrusion of membrane ruffles. ADF/Cofilin activities are downregulated through LIM Kinase to increase the stability of F-actin filaments to facilitate the final engulfment of bacteria. Subsequent to the stimulation of Cdc42 and Rac-1, *Salmonella* delivers another effector protein, SptP, which reverses the activation of these small G proteins by stimulating their intrinsic GTPase activity and therefore facilitating actin depolymerization and cell recovery.

2.3. *Role of* Salmonella *actin-modulating proteins in invasion*

Although many pathogens have evolved strategies to modulate the actin dynamics of the host cell cytoskeleton, *Salmonella* is the only one that encodes actin-binding proteins. SipA and SipC, two type III translocated effector proteins, are involved in modulating actin dynamics by directly binding to actin (Zhou et al., 1999b). Based on in vitro biochemical analyses, SipA is capable of decreasing the critical concentration for actin polymerization, inhibiting depolymerization of actin filaments and increasing the bundling activity of T-plastin. These characteristics have led to the hypothesis that SipA affects actin dynamics in cells by initiating actin polymerization

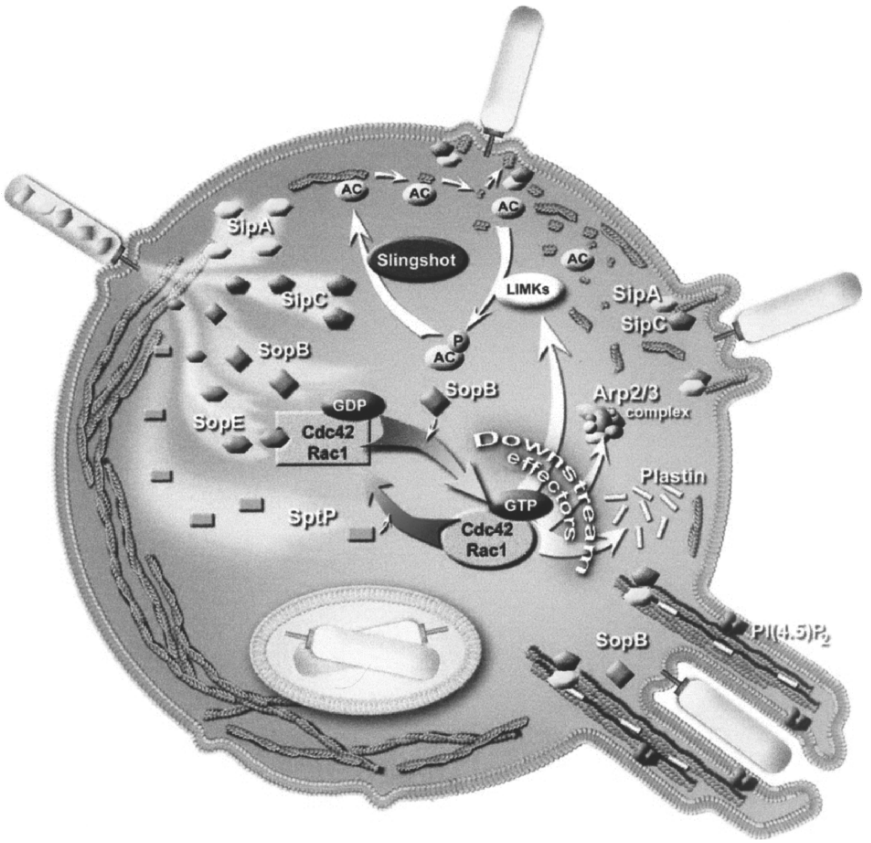

FIGURE 3. Model for *Salmonella typhimurium* interaction with host cells. Contact with host cells activates the invasion-associated type III secretion system, resulting in the delivery of a panel of effector proteins (e.g., SipA, SipC, SopE, SopB, and SptP). Introduction of the exchange factor SopE and the inositol polyphosphatase SopB results in the activation of Cdc42 and Rac-1, the stimulation of downstream signaling pathways including the Arp2/3 complex, and the recruitment of plastin and other ruffling-associated molecules initiating the actin cytoskeleton reorganization. The bacterial effector proteins SipA and SipC help this process by lowering the critical concentration of actin, by stimulating the bundling activity of plastin and stabilizing F-actin, and by nucleating the actin assembly. SopB also helps the membrane fission process by decreasing the local concentration of PIP_2 at the base of the membrane ruffles. The combined activities of these effectors result in a localized and pronounced outward extension of the membrane ruffles, resulting in the engulfment of *Salmonella* in an enclosed membrane compartment. *Salmonella* also delivers another effector protein, SptP, which reverses the activation of these small G proteins by stimulating their intrinsic GTPase activity and therefore facilitating cell membrane recovery. (Contributed by Dr. Clark D. Gedney at the Bio Media Center for Instructional Computing, Purdue University.) (See color insert.)

at the site of *Salmonella* entry by lowering the critical concentration of actin needed for polymerization. Furthermore, SipA may increase the stability of actin bundles that drive and support the growth of membrane ruffles and filopodia, which ultimately engulf and internalize the bacteria by modulating the actin-bundling activity of plastin (Higashide et al., 2002; Zhou et al., 1999a). Studies using cryo-electron microscopy and three-dimensional image reconstruction have revealed that the actin-binding domain of SipA interacts with actin filaments by contacting subdomain 4 of one actin subunit and subdomain 1 of the other actin subunit on the opposite long-pitch helical strand (Galkin et al., 2002). The binding pattern of SipA to actin filaments shares striking similarity to that of nebulin to muscle actin (Galkin et al., 2002). This suggests that SipA interacts with actin filaments by mimicking the host actin-binding protein nebulin. Recently, SipC was found to be able to nucleate and bundle actin in vitro (Hayward and Koronakis, 1999), and SipA can potentiate the nucleating and bundling activities of SipC (McGhie et al., 2001). The coordinated action of SipA and SipC would allow *Salmonella* to initiate prominent localized actin rearrangements at the site of bacteria–host cell contact.

2.4. Salmonella-*induced host cell signaling*

Salmonella entry into epithelial cells results from a series of highly coordinated cellular responses that are triggered by a panel of bacterial proteins delivered into host cells by the T3SS. SopE, SopE2, and SopB, three type III effectors, activate Cdc42 and Rac1 signal transduction pathways to promote actin cytoskeleton rearrangements. SipA and SipC, two *Salmonella* type III secreted actin-binding proteins, directly modulate host actin dynamics to facilitate bacterial uptake. This represents a perfect mimicry of host cellular functions by *Salmonella* effectors to modulate the actin dynamics both directly and indirectly.

Translocated *Salmonella* proteins SopE, SopE2, and SopB activate directly and indirectly the low–molecular weight GTP-binding proteins of the Rho subfamily, in particular Cdc42 and Rac. SopE and SopE2 bind Cdc42 and Rac directly and act as potent exchange factors for these small G proteins (Bakshi et al., 2000; Hardt et al., 1998a; Stender et al., 2000). Interestingly SopB, an inositol polyphosphatase, exhibits overlapping functions with SopE by mobilizing cellular inositol polyphosphate fluxes (Zhou et al., 2001) and controlling levels of phosphatidylinositol-4,5-bisphosphate at the base of *Salmonella*-induced actin rearrangements (Terebiznik et al., 2002) to facilitate ruffle formation. The activation of Cdc42 and Rac triggers a series of signal transduction events that lead to actin cytoskeleton rearrangements and the production of pro-inflammatory cytokines. Biochemical, cellular, and structural studies of SopE have unraveled the molecular mechanism of Cdc42 activation by SopE. *Salmonella* SopE achieves this by binding to the switch I and II region of Cdc42, the same site where the eukaryotic GEFs interact with

(Buchwald et al., 2002) Cdc42. Eukaryotic GEFs exert their GEF catalytic activity through a well-conserved DH domain. Remarkably, the catalytic domain of SopE folds into a completely different structure than the DH domain of all known eukaryotic GEFs (Buchwald et al., 2002).

Cells infected with *S. enterica* serovar Typhimurium quickly recover from the dramatic actin cytoskeleton rearrangements and regain their normal cellular architecture (Takeuchi, 1967). Thus, *Salmonella* has evolved the ability both to induce actin cytoskeleton reorganization and to modulate host cellular signal transduction pathways to help the recovery of the actin cytoskeleton. SptP was found to be partly responsible for this recovery by acting as a GAP for Cdc42 and Rac (Fu and Galán, 1999). Structural analysis indicated that the GAP domain of SptP makes extensive contact with the switch I and II region Rac-1 (Stebbins and Galán, 2000). Residues from SptP directly contact key elements in Rac-1 associated with the GTP hydrolysis reaction, facilitating the hydrolysis of GTP to GDP and thus inactivating Rac-1 (Stebbins and Galán, 2000). SptP may exert its GAP activity toward Cdc42 through a similar mechanism. Similar to the structural analysis of SopE, SptP appears to have evolved convergently from the coevolutionary selective pressures of the host–pathogen interaction.

3. Listeria monocytogenes Invasion

Unlike the multifactor "trigger" entry mechanism, the "zipper" entry mechanism usually requires only a single bacterial factor to engage a host receptor to mediate entry. *L. monocytogenes* is another facultative intracellular pathogen that survives both in the environment and within epithelial cells and macrophages. Two *L. monocytogenes* surface-exposed proteins, internalin InlA and InlB, have been studied in detail for their roles in mediating invasion by binding to host-cell surface receptors. Once inside the host cell (epithelial or macrophage), the bacteria disrupt the vacuolar membrane through the bacterial encoded listeriolysin O (Geoffroy et al., 1987; Mengaud et al., 1987). Following escape of the vacuolar membrane, *L. monocytogenes* moves in the host-cell cytoplasm and spreads from cell to cell (Havell, 1986; Mounier et al., 1990; Tilney and Portnoy, 1989). This section reviews the current understanding of the molecular mechanism of *L. monocytogenes* entry into epithelial cells.

3.1. The Internalin Gene Family

It has been demonstrated that *L. monocytogenes* is capable of entering nonphagocytic cells using both in vitro tissue-cultured cells and in vivo animal studies (Gaillard et al., 1987; Racz et al., 1970). Two bacterial proteins, InlA and InlB, have been demonstrated to play major roles in mediating *L. monocytogenes* entry into nonphagocytic cells. InlA and InlB were originally identified by screening a transposon library of Tn*1545* mutants that failed to enter into cultured human intestinal epithelial Caco-2 cells (Gaillard et al.,

1991). Mutants that failed to enter Caco-2 cells had transposon insertions upstream two open reading frames, *inlA* and *inlB*. Mutations in either *inlA* or *inlB* result in reduced level of invasion in human intestinal epithelial cell line Caco-2 (Dramsi et al., 1995; Gaillard et al., 1991). An *inlAinlB* double mutant maintains very low levels of invasion, illustrating both the key role of InlA/InlB in *Listeria* invasion and possible involvement of other bacterial factors mediating the residual invasion into host cells (Dramsi et al., 1995; Gaillard et al., 1991). Further studies have established that InlA and InlB are capable of conferring invasion to noninvasive and nonpathogenic *L. innocula* when expressed from a plasmid (Dramsi et al., 1995; Gaillard et al., 1991). In addition, beads coated with InlA or InlB are able to invade nonphagocytic cells, indicating that InlA and InlB are sufficient for inducing invasion.

Genes that encode InlA and InlB are arranged in a single operon on the *L. monocytogenes* chromosome. The *inlA* gene encodes an 800 amino acid protein with a typical signal sequence that targets it to the bacterial surface (Figure 4). Some striking features of the InlA protein include: (1) repeats A, which are the leucine-rich repeats (LRRs) made of 15 highly conserved tandem repeats of 22 amino acids with a periodicity of leucine residues; (2) repeats B, consisting of three successive repeats, with two 70 amino acids and one 49 amino acids long; (3) an LPXTG region followed by a hydrophobic sequence at the very C-terminus. The LRR region is critical for inducing bacterial entry and is characteristic of eukaryotic and prokaryotic proteins involved in protein–protein interactions (Kobe and Deisenhofer, 1995). The LPXTG region is responsible for anchoring the InlA protein to the bacterial cell wall through the activity of *L. monocytogenes* sortase presumably in a similar fashion as the anchoring of protein A from *Staphylococcus aureus* (Garandeau et al., 2002; Navarre and Schneewind, 1999).

InlB shares significant sequence similarity and organization with that of InlA (Figure 4). The *inlB* gene is cotranscribed with *inlA* and encodes a 630 amino acid protein. Like InlA, InlB possesses an N-terminal signal sequence

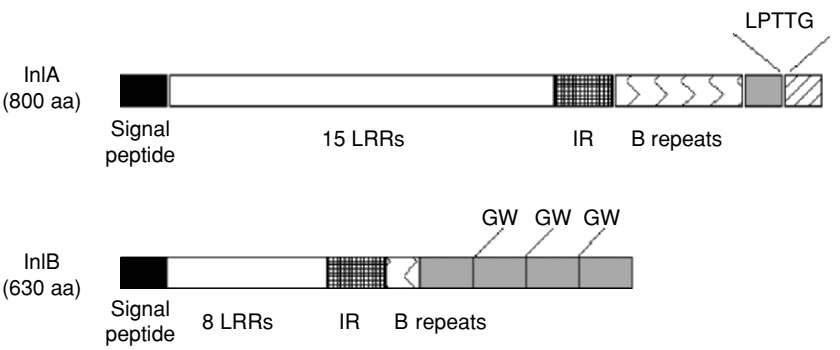

FIGURE 4. Schematic representation of InlA and InlB.

followed by 6 LRRs. It lacks the hydrophobic region in InlA, but has three tandem repeats that start with the dipeptide Gly-Trp, termed GW modules. These GW modules are essential for anchoring InlB on the bacterial surface (Braun et al., 1997; Gaillard et al., 1991). This anchorage is reported to be noncovalent and loose, as InlB is found in the culture media (Jonquieres et al., 1999). Structural analysis of the LRR fragment from InlB has shown that this region adopts an elongated and curved structure that results in exposing a large surface area that may be important in mediating interactions with the corresponding binding partners. Recent work has demonstrated that the LRR region from InlB is not only required for mediating *L. monocytogenes* entry but is also sufficient for the entry process. This strongly suggests that the LRR region is responsible for engaging the host receptor(s) and is critical for subsequent entry.

Based on sequence analysis, there are at least seven additional internalin genes, termed *inlC*, *inlC2*, *inlD*, *inlE*, *inlF*, *inlG*, and *inlH* (Dramsi et al., 1997; Engelbrecht et al., 1996, 1998; Raffelsbauer et al., 1998). The importance of *inlC*, *inlE*, *inlG*, and *inlH* in *L. monocytogenes* pathogenesis has been demonstrated by using a mouse model of listeriosis. However, no major roles in invasion have been demonstrated for these genes using tissue-cultured cell lines. The *inlF* and *inlC2DE* genes have unknown roles in *L. monocytogenes* virulence. Unlike InlA and InlB, the other internalins alone are not sufficient to mediate bacterial or latex beads internalization. All internalin proteins contain the LRR region described above. Most internalins, except InlB and InlC, also possess the LPXTG motif at their C-terminal region, suggesting that they are associated covalently to the bacterial cell wall in a similar fashion as InlA.

3.2. *Host Factors Involved in* Listeria monocytogenes *Entry*

Entry of *L. monocytogenes* into host epithelial cells involves no dramatic membrane reorganization on the host cell surface (Figures 5 and 6; Cossart et al., 2003). This is also true for latex beads covalently coated with internalins. Although there is no membrane ruffling during *L. monocytogenes* entry, the invasion process requires the rearrangement of the actin cytoskeleton beneath the host cell plasma membrane. This has been supported by findings that entry of *L. monocytogenes* is dependent on actin polymerization and is inhibited by cytochalasin D (Gaillard et al., 1987). The first host receptor identified for *L. monocytogenes* invasion was human E-cadherin. Human E-cadherin was identified as binding to InlA using affinity chromatography (Mengaud et al., 1996). Human E-cadherin belongs to a family of transmembrane proteins, termed cell-adhesion molecules (CAMs), which are involved in calcium-dependent cell–cell and cell–extracellular matrix interactions. The extracellular domain of E-cadherin is rigidified by calcium binding and forms

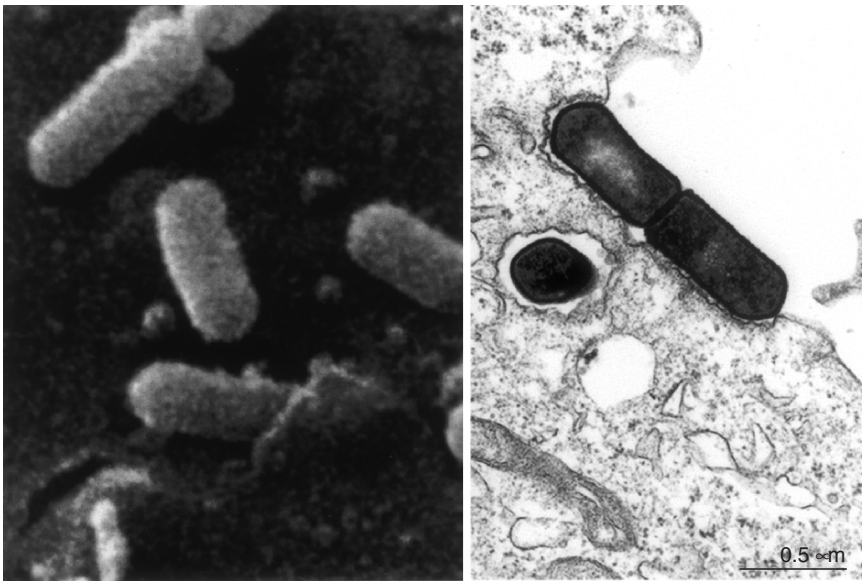

FIGURE 5. Entry of *Listeria monocytogenes* into human Caco-2 cells. (Adapted with permission from Cossart et al., 2003.) (A) Scanning electron micrograph. (B) Thin section. (Reprinted with permission from Elsevier.)

parallel homodimers conferring side-to-side interaction between homodimers. Once formed, the cytoplasmic domains of these E-cadherin homodimers bind adaptor molecules such as beta-catenin, which allows subsequent binding to alpha-actinin or alpha-catenin. The formation of these complexes provides the link to the cortical actin MFs, which is important for the structural and functional integrity of tissues. Studies using various truncations of the cytoplasmic domain of E-cadherin have shown that the linkage of E-cadherin to the actin cytoskeleton is required for InlA-mediated *Listeria* entry (Lecuit et al., 2000). However, questions remain as to how the interaction with E-cadherin, which normally confers cellular adhesion, leads to *Listeria* entry. It has been speculated that a combination of free-moving bacteria and the distribution of InlA on the bacterial surface might have resulted in the zipping of the E-cadherin-containing membrane against the *Listeria* cell body (Cossart et al., 2003).

An important feature of epithelial cells in vivo is the differential distribution of proteins on their apical and/or basolateral surfaces, referred to as polarization. In fully polarized enterocytes, which are the absorptive cells of the intestinal epithelium, E-cadherin is found only at the cell–cell junctions and at the basolateral side of the cell surface. Invasion experiments using polarized Caco-2 cells have indicated that *L. monocytogenes* preferentially invade through the basolateral side and not the apical side (Gaillard and

Finlay, 1996). This raises the question as to how *L. monocytogenes* get across the intestinal epithelial barrier during an infection. Two hypotheses have been proposed suggesting that *L. monocytogenes* does not initiate the infection by invading the apical side of the intestinal epithelial layers. *L. monocytogenes* may infect M cells, which are specialized antigen-sampling cells, to gain access to the basolateral side of the enterocytes in the intestinal epithelial layer. Alternatively, *L. monocytogenes* may invade the host cell through transient openings of the cell–cell junctions.

Crystal structure of the functional domain of InlA and its complex with human E-cadherin has recently been reported (Schubert et al., 2002). It was shown that the LRR domain of InlA surrounds and makes contact with the sixteenth proline amino acid residue of human E-cadherin. In human E-cadherin, this proline amino acid residue defines the specificity for InlA and is replaced by a glutamic acid residue in mouse and rat E-cadherin (Lecuit et al., 1999). As a consequence, wild-type mouse and rat E-cadherins are not receptors for InlA. Replacement of the glutamic acid residue with a proline residue allowed interaction between InlA and the mutated E-cadherin (Lecuit et al., 1999). This discovery solved a long-term mystery as to why wild-type *Listeria* does not cross the mouse intestine efficiently. Subsequent studies using transgenic mice expressing human E-cadherin in the mouse intestine have further established that InlA is indeed essential for *Listeria* entry and crossing of the intestinal barrier (Lecuit et al., 2001).

Two major host cell receptors have been identified for InlB. One is the globular head of the complement component C1q receptor (gC1q-R) (Braun et al., 2000). The other is the Met tyrosine kinase (Met), which is a receptor for heptocyte growth factor (HGF) (Shen et al., 2000) (Figure 6). In addition, it has been reported that InlB also interacts with host cell surface glycosamines (GAGs) (Jonquieres et al., 2001). gC1q-R is a ubiquitous 33 kDa acidic membrane protein that binds to the globular heads of C1q, a component of the complement system. Evidence that supports a role for gC1q-R in InlB-mediated invasion includes: (1) purified recombinant InlB binds gC1q-R protein; (2) C1q, the nature ligand for gC1q-R, competes with InlB for binding to gC1q-R and inhibits *L. monocytogenes* entry into Vero cells; (3) antibodies against gC1q-R inhibit *L. monocytogenes* entry into Vero cells; (4) gC1q-R-transfected GPC16 guinea pig epithelial cells, which do not express gC1q-R, confer the entry of InlB-coated beads; and (5) antibodies against gC1q-R reduce the activation of InlB-mediated PI 3-kinase and tyrosine activation of Gab1, Cbl, and Shc.

The Met tyrosine kinase was identified as the second receptor for InlB (Shen et al., 2000). Met is a heterodimeric membrane-spanning protein made of the 45 kDa α subunit and the 145 kDa β subunit (Bottaro et al., 1991). The natural ligand for Met is the HGF, which is also known as scatter factor due to its ability to cause movement or "scattering" of monolayer epithelial cells upon interaction with Met (Bottaro et al., 1991). The extracellular domain of the α and β heterodimer is involved in binding HGF, and the cytoplasmic

domain of the β subunit possesses the tyrosine kinase activity. Interaction of HGF and Met leads to the autophosphorylation of Met and leads to the phosphorylation and recruitment of downstream signaling molecules.

Participation of Met in InlB-mediated invasion of *L. monocytogenes* seems logical and is further collaborated by the following experimental data: (1) purified InlB protein stimulates the tyrosine phosphorylation of Met; (2) Met is recruited and phosphorylated at the site of *Listeria* entry; (3) Met binds the InlB protein and *L. monocytogenes* strains that express *inlB*, but not to strains that are deleted for the *inlB* gene; (4) Met expression is required for InlB-mediated entry into T47D Met-deficient cell line; and (5) entry into Vero cells is inhibited by either an antibody against an epitope in the InlB-binding domain of Met or a soluble Met fusion protein.

4. Conclusions

Although *Salmonella* and *Listeria* utilize very different molecular mechanisms to gain entry into host cells, they both employ the same fundamental strategy: functional mimicry of key host cellular factors. It is striking that neither primary amino acid sequences nor the three-dimensional structures are effective predictors of functional mimicry. These novel functional mimicry factors have most likely evolved through the long and intimate interactions between bacteria and their host cells. One of the most obvious advantages of functional mimicry is to allow the host cell to regain normal functions after bacterial entry through existing cellular regulatory pathways.

It is becoming evident that *Salmonella* entry into nonphagocytic cells is the result of functional mimicry of a group of bacterial effectors delivered into the host cell by the type III protein secretion system. The concerted action of these effectors allows a balanced approach enabling bacterial entry and host epithelial cell integrity, which is vital for maintaining normal host functions. SopE, SopE2, and SopB intercept the host cell signal transduction pathways to promote actin reorganization. SptP participates in cell recovery by antagonizing the activities of SopE. SipA and SipC act as actin-binding/modulating proteins to further enable the actin rearrangements. The molecular mechanisms of this functional mimicry are further illustrated by a series of structural analyses of SipA, SopE, and SptP, suggesting that these bacterial effectors are novel actin cytoskeleton modulators coevolved from the selective pressures of the host–pathogen interaction.

Listeria uses internalin InlA and InlB primarily to enter host cells through receptor-mediated endocytosis. The identification of multiple receptors for InlB prompted speculations as to whether there are functional overlaps between gC1q-R and Met. There are also suggestions that multiple receptors may enable *L. monocytogenes* to invade different cell types by engaging distinct receptors expressed in a variety of tissues that it encounters during a natural infection. It is also possible that coordination between

these receptors allows more efficient entry in vivo. The engagement of Met by InlA and E-cadherin and of gC1q-R by InlB represents another example of functional mimicry to allow bacteria to enter nonphagocytic cells.

Questions to Consider

1. What are the "trigger" and "zipper" bacterial entry mechanisms?

The "zipper" mechanism involves the engagement of a bacterial ligand with a host cell receptor leading to the gradual movement of the plasma membrane alongside the surface of the invading bacteria and eventually wraps the bacteria inside the host cell membrane. The "trigger" mechanism induces dramatic actin cytoskeleton rearrangements and membrane ruffling leading to macropinocytosis and bacterial entry. While the "trigger" mechanism often involves multiple bacterial effectors, one bacterial factor is usually required and sufficient for inducing bacteria entry by the "zipper" mechanism.

2. What is functional mimicry?

Functional mimicry describes the bacterial determinants that perform certain functions of host cellular factors without having similarities of primary amino acid sequences, or overall resemblance of three-dimensional structures. These novel functional mimicry factors are likely evolved through the long and intimate interactions between the bacteria and their host cells.

3. Define cytoskeleton.

The cytoskeleton is a structure that is responsible for cell movement, cytokinesis, and the organization of the organelles within the cell. Three types of cytoskeleton components are microfilaments (MF), microtubules (MT), and intermediate filaments (IF), which exist to provide both movement and stability in mammalian cells. Each of these cytoskeleton structures is dynamic in nature and composed of polymers of subunits. MFs are assembled from monomers consisting of actin; MTs from tubulin; and IFs from IF proteins. In nonphagocytic epithelial cells, MFs project into the villi, giving shape to the cell surface. MTs grow out of the centrosome to the cell periphery. IFs connect adjacent cells through desmosomes.

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Chapter 9

Bacterial Protein Secretion Mechanisms

JAMES W. WILSON

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Historical Landmarks

1981 The first *secA* mutant is isolated, representing a major step in the characterization of the general secretory (or "Sec") pathway (Oliver and Beckwith, 1981).

1985 The *Escherichia coli* alpha-hemolysin ABC export system is the first identified type I protein secretion system (Felmlee et al., 1985a,b).

1987 Pugsley and colleagues perform the first characterization of the type II pathway while studying the secretion of the *Klebsiella oxytoca* pullanase enzyme (d'Enfert et al., 1987).

1987–1989 The IgA1 proteases of *Neisseria* and *Haemophilus* are the first proteins shown to be secreted by the autotransporter mechanism (Pohlner et al., 1987; Poulsen et al., 1989).

1990 The discovery of the type III pathway occurs with the observation that *Yersinia* Yop proteins are not outer membrane proteins (OMPs) as initially proposed, but are secreted into the external media via a novel mechanism (Michiels et al., 1990).

1996 The existence of the twin-arginine secretion pathway is proposed (Berks, 1996).

1997 The relationship between the *Agrobacterium* T-DNA transfer system and other protein secretion systems is identified, and the large family of type IV secretion systems (T4SS) is established (Christie, 1997).

1. Introduction

1.1. Protein Secretion Mechanisms Are Essential for the Interaction of Bacteria with Host Cells

All bacteria have mechanisms to export proteins from the cytoplasm to the cell envelope or to the extracellular environment. For bacterial species that live in close contact with eukaryotic hosts, including pathogens, the ability to perform this task is of critical importance because interactions with host cells involve proteins that must be secreted from the bacterial cell and displayed extracellularly or translocated to the host cell cytosol. Extracellular structures can range from single proteins anchored to the cell envelope to elaborate, multiprotein structures such as pili, fimbriae, and protein secretion/translocation needles. In addition, all of the classic bacterial toxins must be secreted from the bacterial cell as a requisite step in interacting with the host cell, and the ability to exchange genetic information (including virulence genes) between bacterial cells is dependent on protein secretion. Clearly, the mechanisms of protein secretion form a cornerstone in building an understanding of bacterial survival and pathogenesis.

1.2. There Are Sec-dependent and Sec-independent Secretion Pathways

For both Gram-positive and Gram-negative bacteria, the major route for protein secretion is the general secretory pathway or "Sec" system. This secretion pathway is involved with the insertion of most membrane proteins and is the main mechanism for crossing the bacterial plasma membrane. (For Gram-positive bacteria, this is the only membrane barrier at the cell envelope, while for Gram-negative bacteria, the plasma membrane corresponds to the inner membrane.) The Sec systems of Gram-positive and Gram-negative bacteria are highly related at both the sequence and mechanistic levels (Economou, 1999; van Wely et al., 2001). In addition to the Sec pathway, alternative secretion pathways are present in both Gram-positive and Gram-negative bacteria. Most of the alternative secretion mechanisms in Gram-negative bacteria have evolved different ways for the protein substrates to transverse the outer membrane. Some of the alternative mechanisms in Gram-negative bacteria use the Sec pathway for crossing the inner membrane and then use a separate pathway for crossing the outer membrane. These are referred to as "Sec-dependent" secretion pathways where the protein substrate crosses the cell envelope in *two steps*. Other Gram-negative mechanisms use a pathway that does not involve the Sec system for secretion of substrates across both the inner and outer membranes. These mechanisms are termed "Sec-independent" secretion pathways where *one step* is used for transversal of both membranes. However, variations on this dichotomy exist. There is a Gram-negative secretion mechanism that secretes both Sec-dependent and -independent substrates (the type IV pathway). In addition, there are also Sec-independent pathways for moving proteins across the plasma/inner membrane in Gram-positive and Gram-negative bacteria (the twin-arginine pathway and the Gram-positive ABC transporters). Known pathways for bacterial protein secretion that have been characterized to date are presented in Figure 1.

The topic of bacterial protein secretion mechanisms is a vast one, and it is based on a large body of experimental data accumulated by many dedicated scientists. Some of the references for individual sections of this chapter are reviews that contain much of the presented information for that section. Additional, specific references (if not provided here) can be found within those reviews. For simplicity and space constraints, when a section or paragraph explains specific experimental findings, the references for those findings have been cited within the first few sentences of that section or paragraph.

2. The Sec System (General Secretory Pathway)

The Sec systems in Gram-positive and Gram-negative bacteria are highly related (Economou, 1999; van Wely et al., 2001). The Gram-negative Sec system will be discussed first to "lay the groundwork" for the description of this system

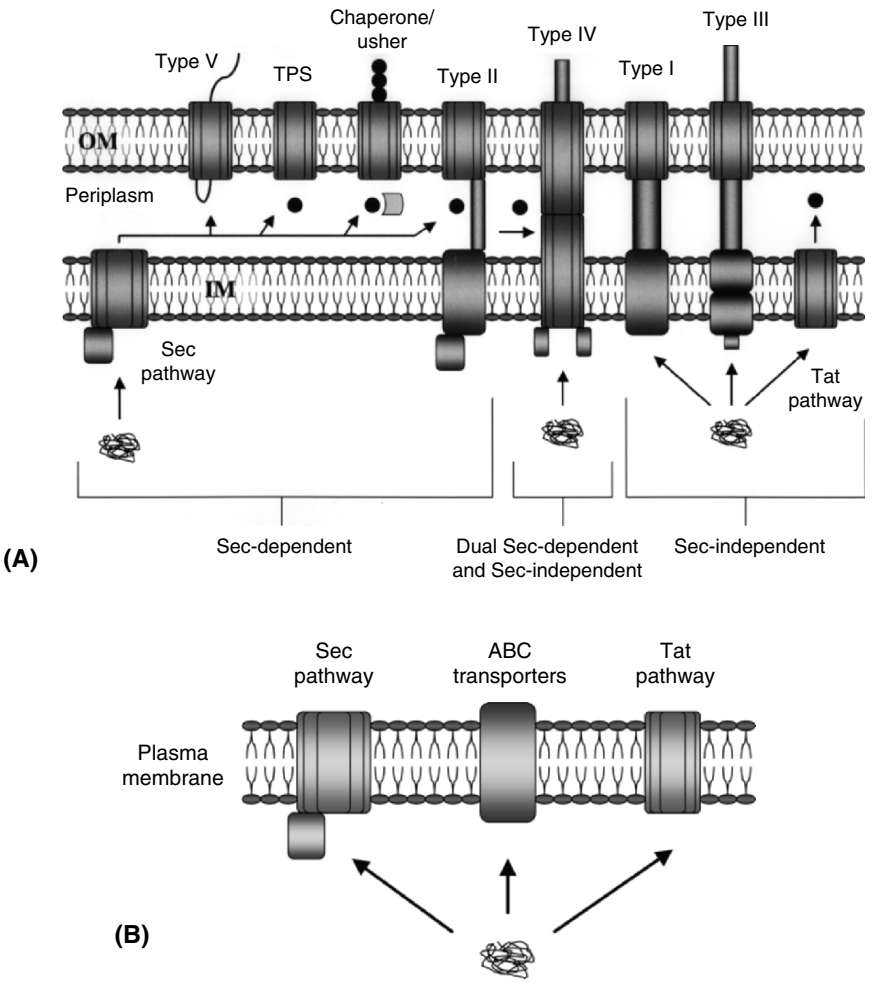

FIGURE 1. Pathways for bacterial protein secretion. (A) Protein secretion pathways in Gram-negative bacteria. The major protein secretion pathways in Gram-negative bacteria are depicted as Sec-dependent, Sec-independent, and dual Sec-dependent and Sec-independent mechanisms. Note that the Sec-independent substrates each contain specific signals that "tag" the protein for secretion via each different pathway, though one substrate is depicted here for simplicity. (B) Protein secretion pathways in Gram-positive bacteria. The major protein secretion pathways in Gram-positive bacteria are depicted. Note that the substrates for each pathway contain specific signals that "tag" the protein for recognition by each pathway, though one substrate is depicted here for simplicity.

followed by a list of the differences between the Gram-negative and Gram-positive Sec systems. Most of the experimental data to support our current understanding of the Sec system has been obtained from studies in *Escherichia coli* (for Gram-negative cells) and *Bacillus subtilis* (for Gram-positive cells). It is commonly thought that conclusions made from these particular systems apply to most bacteria in each group due to both empirical evidence from other systems and the highly conserved nature of the Sec system among all cells.

2.1. The Gram-negative Sec System

2.1.1. The Major Components of the Sec System

2.1.1.1. SecA

The SecA protein associates with the Sec machinery on the cytoplasmic side of the inner membrane. It is an essential protein and plays a central role in directing Sec-dependent transport. SecA contains separate domains involved with: (1) binding proteins destined for secretion (called "preproteins"); (2) ATP binding and hydrolysis; and (3) association with the inner membrane. Utilizing energy from both ATP binding and ATP hydrolysis, SecA undergoes conformational changes that serve to "push" the preprotein into a membrane channel formed by SecY, E, and G and into or across the membrane (de Keyzer et al., 2003; Economou, 1999, 2002; Mori and Ito, 2001).

2.1.1.2. SecB

The SecB protein is a chaperone that binds certain preproteins and targets them for Sec-dependent secretion by facilitating their interaction with SecA (Randall and Hardy, 2002). It is not an essential protein and other chaperones (such as DnaK and GroEL) are thought to substitute for SecB in certain cases. However, many secreted proteins require SecB for proper export.

2.1.1.3. SecY, SecE, and SecG

These three proteins form a channel in the membrane through which the Sec-dependent proteins are secreted (de Keyzer et al., 2003; Economou, 1999, 2002; Mori and Ito, 2001). This channel is termed the "translocase." These proteins are polytopic membrane proteins with several membrane-spanning domains and together form a ringlike structure that can be visualized by electron microscopy (Manting et al., 2000; Meyer et al., 1999). The complex of SecA and SecY, E, and G forms the core molecular machine that directs secretion.

2.1.1.4. SecD and SecF

The SecD and SecF proteins are inner membrane polypeptides with large domains located in the periplasm (SecY, E, and G do not have such domains) (Economou, 2002). SecD and SecF are most likely involved with late stages

of secretion, such as preprotein release, and with regulating the insertion cycles of SecA during secretion. A small protein, YajC, assembles into a complex together with SecD and SecF. In Gram-positive bacteria, a single polypeptide known as SecDF can serve as the functional equivalent of SecD and SecF (Tjalsma et al., 2004; van Wely et al., 2001).

2.1.1.5. YidC

This protein associates with the Sec translocase and is involved with the proper insertion of certain inner membrane proteins, such as respiratory chain complexes and cytochromes (Dalbey and Kuhn, 2004; de Gier and Luirink, 2003). YidC also plays a role in the assembly of some inner membrane proteins, which do not require the Sec system for their membrane insertion.

2.1.1.6. Ffh, 4.5S RNA, and FtsY

The complex of the Ffh protein (a GTPase) and the small 4.5S RNA form what is known as the "signal recognition particle" (SRP) (de Keyzer et al., 2003; Economou, 2002). As its name implies, this complex recognizes the signal sequence on certain Sec-dependent proteins that target them for secretion (see below). The binding of SRP to the preprotein arrests its translation until it can be engaged to the secretion machinery. The prokaryotic SRP is very similar to its eukaryotic SRP counterpart, and Ffh can functionally replace its eukaryotic homologue for SRP formation, signal peptide recognition and translation arrest (but not for preprotein translocation) (Herskovits et al., 2000). The Ffh and 4.5S RNA SRP binds to a membrane-associated protein named FtsY for targeting to the inner membrane.

2.1.1.7. Signal Peptidases I and II

Signal peptidases I and II are membrane-bound proteins that proteolytically process secreted substrates at certain sites in their protein sequences (Paetzel et al., 2002). Signal peptidase I (also known as "Lep") serves to cleave the signal sequence from secreted substrates to form the mature, secreted protein. Signal peptidase II (also know as "LspA") is involved in the processing of lipoproteins and cleaves the lipoprotein signal sequence from certain secreted proteins to facilitate their attachment to membrane lipids.

2.1.2. Secretion via the Sec System Can Be Divided into Three Steps: Targeting, Transmembrane Crossing, and Release

2.1.2.1. Targeting

Sec-dependent secretion involves the recognition of signals in the nascent protein.

The Signal Sequence. Nascent proteins destined for Sec-dependent secretion contain certain signals that are recognized by the Sec-associated machinery.

One such signal is a short, amino terminal sequence called the "signal sequence." It consists of 18–26 amino acids and starts with the first amino acid of the protein (Fekkes and Driessen, 1999). The first 1–5 residues have a net positive charge (the N-domain); the next 7–15 residues make up a hydrophobic region (the H-domain); and the next 3–7 amino acids indicate the cleavage signal that precedes the actual cleavage site (the C-domain). Signal sequence (or "leader") peptidases cut the protein at the cleavage site, and the mature protein is released from the signal peptide to the periplasmic environment or to be inserted into the membrane. Signal peptidase I (Lep in *E. coli*) cleaves most common Sec-dependent substrates, while signal peptidase II (LspA in *E. coli*) cleaves lipoproteins that are anchored to the membrane by a lipid–protein bond. Some signal sequences are thought to insert into the membrane directly and then initiate subsequent interaction with sec apparatus or mediate membrane insertion by a different mechanism.

Mature Domain in the Secreted Substrate. Another way that preproteins are targeted to the Sec machinery is by recognition of a "mature domain" in the protein by SecB, a cytosolic chaperone that interacts with the Sec system. The mature domain is an unfolded- or partially folded domain in a preprotein that is able to bind to SecB, causing the rest of the protein to remain unfolded (or loosely folded) until secretion. SecB can bind certain preproteins with such domains and bring them to the Sec machinery by interacting with SecA, the central component of the Sec apparatus. SecB then "hands off" the preprotein to SecA and disassociates from the Sec apparatus (Randall and Hardy, 2002).

Individual Proteins May Use Secretion Signals Differently. It is important to remember that different secreted proteins that interact with Sec components may have different secretion signals (de Keyzer et al., 2003; Fekkes and Driessen, 1999). Some proteins use the SRP for recognition of the signal sequence. However, some proteins that have a signal sequence can utilize SecB for recognition as well in a signal-sequence independent manner. Some proteins rely solely on SecB for secretion and do not engage the SRP efficiently. Certain proteins are thought to utilize the SRP for membrane targeting and then become inserted via a Sec-independent mechanism, while there is evidence that others may interact with the Sec system without engaging either the SRP or SecB. Figure 2 summarizes these different possibilities. The general view of Sec-targeting is that Sec-dependent inner-membrane proteins utilize the SRP mechanism (sometimes with assistance from YidC), while Sec-dependent secreted proteins (including outer-membrane proteins) are more likely to utilize the SecB mechanism.

2.1.2.2. Transmembrane Crossing: The SecA Cycling Model

In the SecA cycling model, SecA serves as a motor that drives protein secretion via its ATPase activity and from the transmembrane proton gradient (Figure 3) (de Keyzer et al., 2003; Economou, 2002; Mori and Ito, 2001). SecA associates with the SecYEG translocase on the cytoplamic side of the

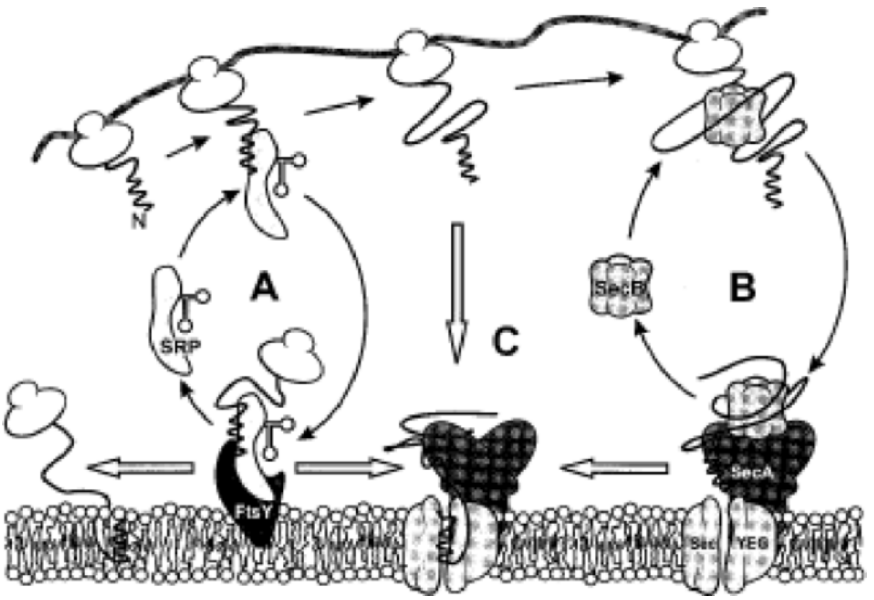

FIGURE 2. Different routes for targeting a protein for membrane insertion or secretion. (A) The signal sequence of a preprotein or integral membrane protein can be recognized by the SRP and targeted to the membrane via interaction with FtsY. At this point, the protein can either be engaged by the Sec apparatus (SecA, SecYEG) or be inserted into the membrane via a different mechanism. (B) Alternatively, when the signal sequence is not recognized by the SRP, the SecB chaperone may bind the mature domain and then target the protein to the Sec apparatus. (C) In some cases where a protein substrate is not recognized by the SRP or SecB, it is possible for the signal sequence to target it directly to the Sec apparatus. (From Fekkes and Driessen, 1999.)

inner membrane, and receives a preprotein from either the SRP or SecB. Once ATP and a preprotein are bound by SecA, the SecA protein undergoes a conformational change that serves to translocate (or "push") the preprotein substrate into the translocase and to the periplasmic side of the membrane. After a single "push" of about 20–30 residues of the preprotein through the translocase, SecA hydrolyzes the bound ATP and reassumes its relaxed conformation. At this point, SecA is thought to transiently dissociate from the preprotein and then reassociate for another round of translocation and ATP hydrolysis. In this way, SecA goes through cycles of conformational changes that translocate the preprotein in 20–30 amino acid intervals. How SecA and SecYEG can work together to secrete such a large number of different proteins (probably hundreds) is remarkable, and the basis for their recognition of such a vast array of substrates remains unknown at this point.

2.1.2.3. Release

After translocation, release of the protein from the translocase occurs (Economou, 2002). This release may involve cleavage of the signal sequence by the signal peptidases, but cleavage may occur soon after translocation initiation for some substrates. Release is also thought to be facilitated by the SecD and SecF proteins via their large periplasmic domains, perhaps to aid in folding of the translocated protein upon export termination. Spontaneous release of a fully translocated protein is also a mechanism that may occur.

2.2. The Gram-positive Sec System

The Gram-positive and Gram-negative Sec systems are almost identical. The mechanism of Sec-dependent secretion described above for Gram-negative bacteria is essentially the same in Gram-positive organisms. However, there are some notable differences:

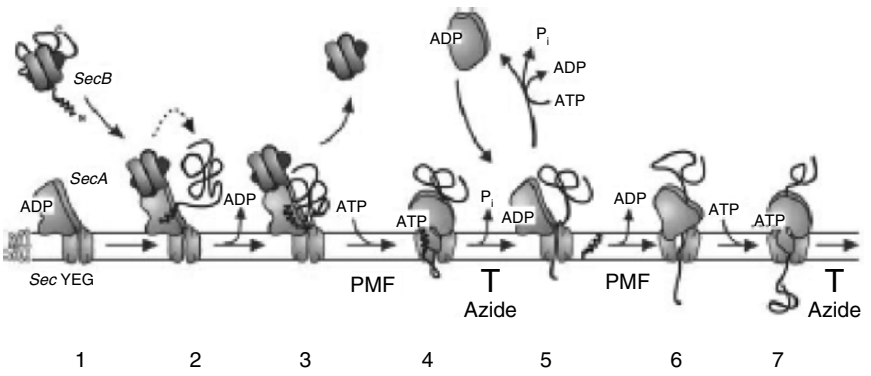

FIGURE 3. Model for SecA-driven protein secretion via cycles of ATP binding and hydrolysis. (1) The Sec translocase consisting of SecA (shown here bound to ADP) and SecYEG is depicted in the plasma membrane. (2) The SecB preprotein complex binds to SecA where SecB "hands off" the preprotein to SecA. Preprotein binding also prepares SecA to exchange ADP for ATP. (3) SecB disassociates from the SecA preprotein complex. (4) SecA binds ATP, which results in a SecA conformational change that drives translocation of approximately 20–30 amino acid residues of the preprotein. (5) ATP hydrolysis results in the release of SecA from the preprotein and retraction of SecA from its "translocation driving" conformation. At this point, SecA can either disassociate from the membrane or rebind the partially translocated preprotein. (6) Rebinding of SecA to the preprotein results in translocation of another 20–30 amino acid residues and allows SecA to bind ATP again. (7) A new round of ATP binding, SecA conformational change, preprotein translocation, and ATP hydrolysis occurs. This cycle continues until the preprotein is fully exported through the translocase pore. PMF indicates steps where the proton motive force is thought to provide energy. Azide indicates steps that are inhibited by the presence of azide. (From de Keyzer et al., 2003.)

(1) The Gram-positive Sec system does not appear to have a SecB component (Economou, 2002; Tjalsma et al., 2004). No homologs with sequence similiarity to the Gram-negative SecB are present in any of the sequenced Gram-positive genomes. Instead, another chaperone called CsaA is functionally very similar to SecB and appears to fulfill SecB-related functions in Gram-positive organisms (Economou, 2002; Tjalsma et al., 2004).
(2) In *B. subtilis* and other Gram-positive bacteria, the SecD and SecF genes are fused (they are adjacent, cotranscribed genes in Gram-negative bacteria) and expressed as a single protein called SecDF (Tjalsma et al., 2004; van Wely et al., 2001). This protein has two easily recognizable SecD and SecF domains.
(3) Gram-positive species have many more signal peptidases and processing enzymes than do Gram-negative bacteria. A particularly important signal peptidase present in many Gram-positive species is a protein called "sortase" (SrtA) (Cossart and Jonquieres, 2000; Mazmanian et al., 2001). This peptidase recognizes a canonical LPXTG motif in the C-terminus of certain secreted proteins, cleaves the protein at this motif, and attaches the protein to the cell wall via the pentaglycine crosslink bridge. Many cell wall–attached proteins that play critical roles in virulence are processed by sortase (Cossart and Jonquieres, 2000; Mazmanian et al., 2001). There is another gene in Gram-positive species that encodes a sortase homolog called SrtB (Bierne et al., 2004). The SrtB peptidase recognizes a different signal motif (NPQTN) for cleavage of its substrates. Another cell wall–anchoring protein, SrtC2, has been identified in *Streptococcus pyogenes* and it recognizes the motif QVPTGV for its processing activity (Barnett et al., 2004).
(4) The Gram-positive cell wall may play a role in the stability or folding of secreted proteins. Mutations in a cell wall synthesis enzyme (Dlt) and a cell wall–associated protease (WprA) have been shown to change protein stability after export (van Wely et al., 2001).

3. Sec-dependent Secretion Systems

The Sec-dependent secretion systems are essentially mechanisms for proteins to cross the outer membrane. Their substrates are proteins in the periplasm that have used the Sec system to cross the inner membrane en route to being exported to the extracellular environment.

3.1. Type V Secretion: Autotransporters

As their name implies, autotransporters self-catalyze their own transport across the outer membrane and represent the most simple and "streamlined" secretion system. This mode of secretion has been termed "type V" secretion in the standard nomenclature, though a recent proposal has suggested

including the two-partner secretion pathway in this group (Desvaux et al., 2004a,b). The autotransporters and the two-partner secretion systems will be presented as distinct mechanisms in this chapter.

A model of type V secretion is given in Figure 4. An autotransporter protein consists of three domains: the N-terminal signal sequence, the passenger domain, and the C-terminal beta-domain (Henderson and Nataro, 2001). The signal sequence is recognized by the Sec system and directs transport of the autotransporter across the inner membrane. The signal sequence is cleaved upon Sec-dependent transport. From the periplasm, the beta-domain

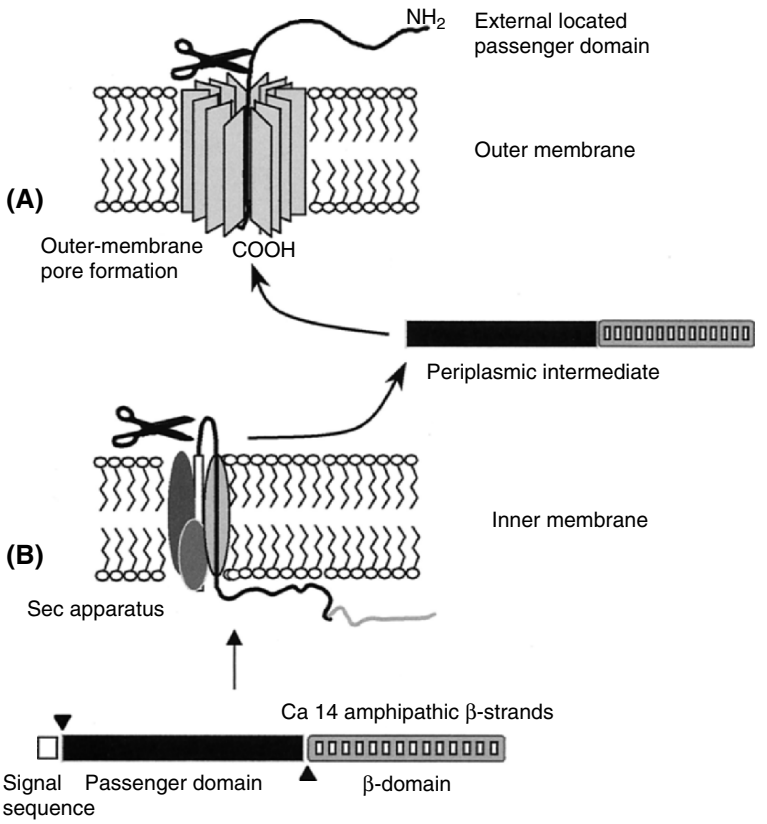

FIGURE 4. A model of type V secretion (autotransporters). Autotransporters are translated as a polyprotein containing three domains: the signal sequence, the passenger domain, and the beta-domain. The signal sequence targets the polyprotein to the Sec apparatus and is cleaved upon export to the periplasm. The beta-domain in the periplasmic intermediate of the autotransporter assumes a beta-barrel for insertion into the outer membrane and pore formation. The passenger domain is then secreted to the outer surface where it may remain attached to the pore or undergo processing. If processing occurs, the passenger domain may be released into the extracellular milieu or remain associated with the bacterial cell surface. (From Henderson and Nataro, 2001.)

inserts into the outer membrane and assumes a beta barrel–shaped structure that forms a membrane pore. The passenger domain is exported to the bacterial cell surface through the beta-domain pore and either is cleaved and released or remains associated with the beta-domain. A large number of virulence factors found in a wide range of Gram-negative species use the autotransporter mechanism for secretion (Henderson and Nataro, 2001). The most important of these are the IgA1 proteases and the serine protease autotransporters of *Enterbacteriacae* (SPATEs).

The "subdomains" of the *Yersinia* YadA autotransporter protein have been characterized in detail (Roggenkamp et al., 2003). The YadA protein is an adhesin that forms unique, oligomeric lollipop-type structures at the cell surface which are membrane-embedded via their C-termini. YadA consists of six different domains: the N-terminal signal sequence, the head, neck, and stalk domains, a linker region, and a C-terminal membrane-anchoring domain. The head, neck, and stalk domains compose the passenger domain of YadA. The unique topology of the YadA protein has led to its designation as the prototype in a subgroup of the autotransporters termed the oligomeric coiled-coils adhesin (Oca) family (Desvaux et al., 2004a,b).

3.2. Two-partner Secretion Pathway

The two-partner secretion (TPS) pathway consists of the secretion substrate protein (generically termed the TpsA component) and a pore-forming outer membrane protein (termed the TpsB component) (Jacob-Dubuisson et al., 2001). The TpsA protein contains a signal peptide and is transported across the inner membrane via the Sec system. Another domain on TpsA, the S domain, targets the protein for secretion across the outer membrane via TpsB. At the cell surface, TpsA proteins may undergo further folding and proteolytic cleavage reactions to complete their processing to mature proteins. A remarkable feature of TpsA preproteins is an N-terminal extension of the signal peptide that stretches about 25 amino acids upstream of the typical N-, H-, and C-domains of the signal sequence. This feature is also found in some large secreted adhesins and autotransporters. It has been hypothesized that this N-terminal extension may be recognized by a "pilot protein" or SRP homolog that targets the protein for Sec-dependent secretion. In addition, some TpsA proteins require a cytoplasmic partner, termed TpsC, for proper secretion. Figure 5 depicts the TPS pathway. The best-known members of the TPS family are the ShlA/ShlB proteins of *Serratia marcescens* and the FHA system of *Bordetella pertussis*.

3.3. Chaperone/usher pathway

The chaperone/usher pathway is very similar to the TPS pathway. It depends on the Sec system for transport of substrates across the inner membrane and utilizes an outer membrane pore protein (or "usher") for crossing the outer

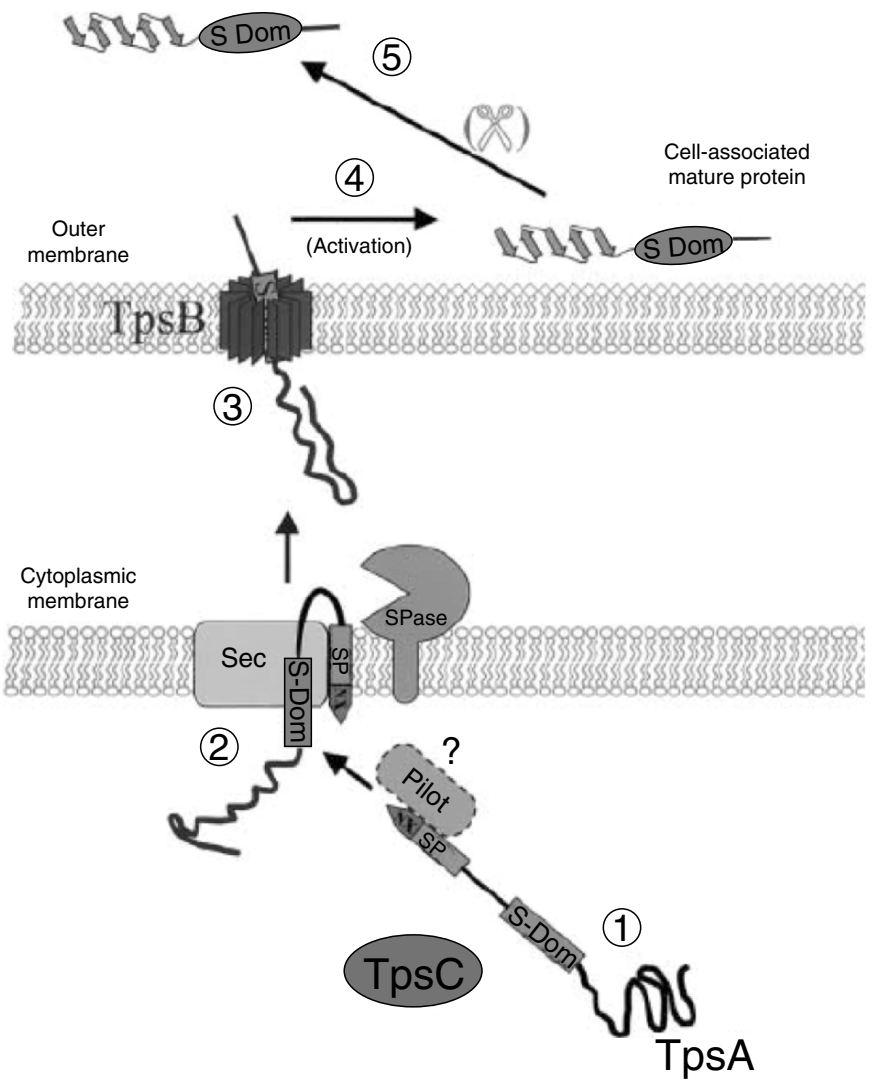

FIGURE 5. The twin-partner secretion (TPS) pathway. (1) The TpsA preprotein substrate is depicted. Some features specific to TpsA proteins are: (i) an N-terminal extension (NX) that precedes the signal sequence (also known as the signal peptide, SP); (ii) an S-domain that targets the protein for secretion via the TpsB component; and (iii) the requirement for a cytoplasmic helper protein, TspC, for proper secretion. It is hypothesized that the N-terminal extension may recruit an additional "pilot" protein to aid in secretion via the Sec apparatus. (2) The TpsA preprotein is exported to the periplasm via the Sec system and cleaved by a signal peptidase. (3) The TpsA protein is secreted through the outer membrane TpsB channel. (4) Folding of the TpsA exoprotein at the cell surface, which results in the activation of TpsA proteins which contain cytolysin activity (such as the ShlA protein of *Serratia marcescens*). (5) Cell-associated TpsA exoproteins can be released at this point, and this may involve possible proteolytic processing. (From Jacob-Dubuisson et al., 2001.)

membrane. However, it also requires a periplasmic chaperone that binds the substrate protein and aids in its export via the usher. This pathway is involved in the secretion and assembly of a wide range of surface structures that often play roles in adherence and other virulence-related functions. The two best-characterized chaperone/usher assembly mechanisms are the P pili and type I pili of *E. coli;* the P pili will be used as the model to explain this secretion mechanism, though all chaperone/usher pathways seem to work almost identically (Fernandez and Berenguer, 2000; Thanassi and Hultgren, 2000; Thanassi et al., 1998).

There are three groups of components that participate in the chaperone/usher pathway: (1) the outer membrane usher; (2) the periplasmic chaperone; and (3) the pilus subunits. The latter group contains the secreted substrates, and there are multiple different substrate proteins making up different subdomains of the pilus (such as the tip, the tip adaptor, and the major pilus subunits). The periplasmic chaperone recognizes and binds these different substrates. A general model of the chaperone/usher pathway (based on the P pilus model) is depicted in Figure 6. Following Sec-dependent export across the inner membrane, the pilus subunits are bound by the periplasmic chaperone. The chaperone interaction is critical and serves to allow release of the substrate into the periplasm, facilitate proper folding, prevent premature subunit interactions in the periplasm, and target the substrate to the outer membrane usher. Once guided to the usher, the substrate is released from the chaperone and is translocated across the outer membrane and incorporated into the growing pilus structure. Formation of the pilus is terminated by incorporation of the PapH subunit, which serves to anchor the pilus and signal the end of the structure formation.

3.3.1. The Usher Protein (PapC) Forms a Selective Outer-Membrane Pore

PapC-like ushers are composed of primarily beta-strands and form oligomeric complexes in the outer membrane that are visible by electron microscopy (Thanassi and Hultgren, 2000; Thanassi et al., 1998). Since the pore of the oligomeric PapC pore is 2–3 nm and the mature pilus rod is about 7 nm in diameter, the growing pilus rod most likely "unravels" to a thin 2–3 nm fiber for passage through the pore and later folds to a helical structure outside the cell (Thanassi and Hultgren, 2000). Unraveled subunits are continually added to the growing structure from the periplasm. PapC has been shown to display different affinities for different PapD/substrate complexes in vitro. Remarkably, the complexes with the highest affinity for PapC are those containing adhesin or tip subunits (those subunits that are at the end of the pilus), and these would be expected to be the first subunits translocated and displayed on the surface of a growing pilus structure (Fernandez and Berenguer, 2000). Once the usher has bound and incorporated the tip subunits, the other chaperone/subunit complexes can bind to the usher and be added to the growing pilus.

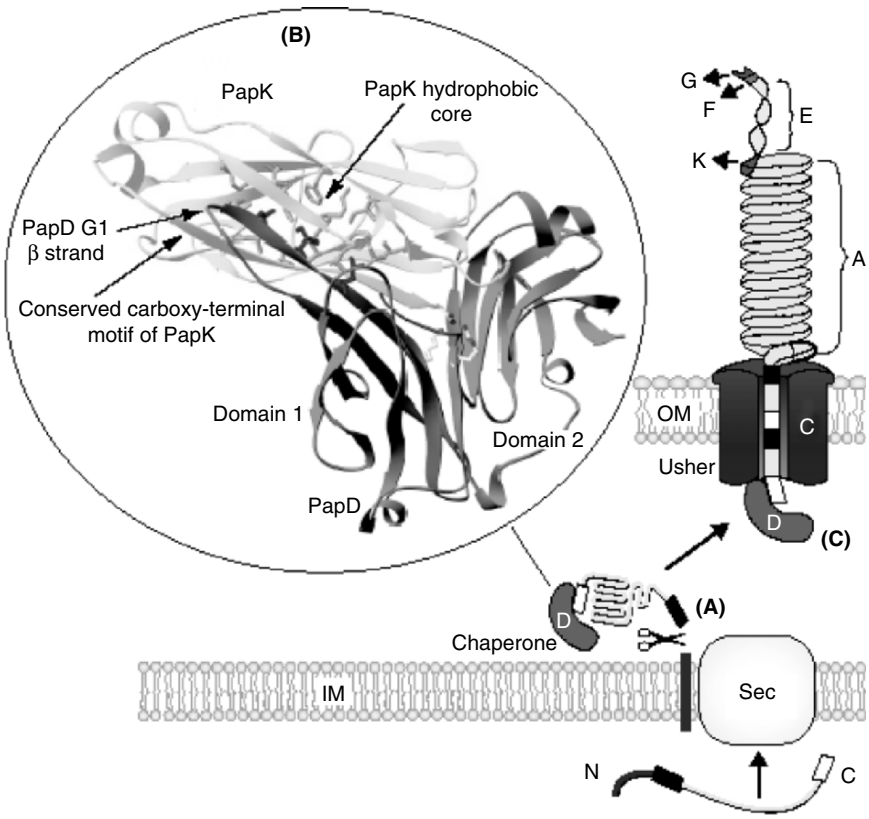

FIGURE 6. The chaperone/usher pathway (depicted using the model for P pili biogenesis). (A) The pilus subunits (PapA, K, E, F, and G) are exported to the periplasm via the Sec system and are processed by signal peptidase. The periplasmic chaperone PapD binds to the subunits to allow proper folding and prevent premature subunit–subunit interactions. (B) The crystal structure of the PapD/PapK complex is shown to illustrate donor strand complementation. The PapK subunit lacks the seventh beta-strand in its Ig fold domain, and this leaves its hydrophobic core exposed. PapD donates the G1 beta strand from one of its two Ig fold domains to complete the structure of the PapK subunit. (C) The chaperone/subunit complex is targeted to the outer-membrane usher component, which secretes the subunit across the outer membrane and allows assembly of the pilus structure. The pilus subunits interact and polymerize via a donor strand exchange mechanism where the N-terminal extension of one subunit displaces the donated G1 strand of the chaperone and completes the Ig domain with the other subunit. An array of subunit Ig domains is formed. (From Thanassi and Hultgren, 2000. Reprinted with permission from Elsevier.)

3.3.2. The Chaperone Protein (PapD) Binds and Protects Pilus Subunits via a "Donor Strand Complementation" Mechanism

The pilus subunits that are substrates for the chaperone/usher pathway have Ig fold domains, which lack a seventh C-terminal beta-strand that is present in the canonical Ig fold structure (Sauer et al., 1999; Thanassi and Hultgren, 2000). As a result, a deep, hydrophobic groove in the subunit is exposed. The chaperone protein, PapD, consists of two Ig domains connected by a peptide linker. When PapD binds individual subunits, it positions (or "donates") one of its Ig domain beta-strands (the G1 strand and a portion of the F1–G1 loop) to complete the Ig fold in the subunit protein and thereby shield the hydrophobic groove of the subunit. This mechanism of binding, termed donor strand complementation, stabilizes the subunit and prevents premature subunit polymerization in the periplasm.

3.3.3. Pilus Assembly Involves a "Donor Strand Exchange" Mechanism

The pilus subunits have an N-terminal extension, which is not part of the Ig domain and lacks a rigid structural organization in the crystal structure (Sauer et al., 1999; Thanassi and Hultgren, 2000). When the chaperone/subunit complex is brought to the usher during pilus assembly, the N-terminal extension of one subunit displaces the donated G1 beta strand of the chaperone of another subunit, and the two subunits bind by completing their Ig folds together. This process, termed donor strand exchange, allows the entire pilus structure to polymerize as an array of subunit Ig domains.

Additional description of the chaperone/usher pathway is given in Chapter 7 on bacterial adhesins.

3.4. Type II Secretion Pathway

The type II secretion pathway is the most complex Sec-dependent pathway and has been referred to as the "main terminal branch of the general secretory pathway" (Sandkvist, 2001a,b). As compared to the type V, TPS, and chaperone/usher pathways, many more individual protein components are involved in the functioning of the type II pathway. The type II pathway is found in a wide variety of Gram-negative species and is often used to secrete virulence factors (Sandkvist, 2001a,b). Examples of the type II pathway include the *Klebsiella oxytoca* pullulanase secretion system (which was the first discovered type II pathway) and the *Vibrio cholera* toxin secretion system. The individual protein components of this pathway have been assigned letters (A through O) in order to standardize their identification across species.

A diagram of type II secretion is given in Figure 7. Substrates targeted for secretion first cross the inner membrane via the Sec system. The substrate illustrated in Figure 7 is a typical A-B toxin, such as cholera toxin.

Following assembly of the AB5 toxin in the periplasm, the toxin is then recognized by the type II machinery via a signal in the B5 subunits. The type II apparatus forms a pilus-type structure (with protein G as the main subunit), which is thought to "push" the toxin through an outer membrane pore in a piston-like manner. However, other models exist where a complex of multiple type II outer membrane pores are utilized for pilus extension and retraction (which provide a driving force for secretion) and toxin export (Sandkvist, 2001a,b).

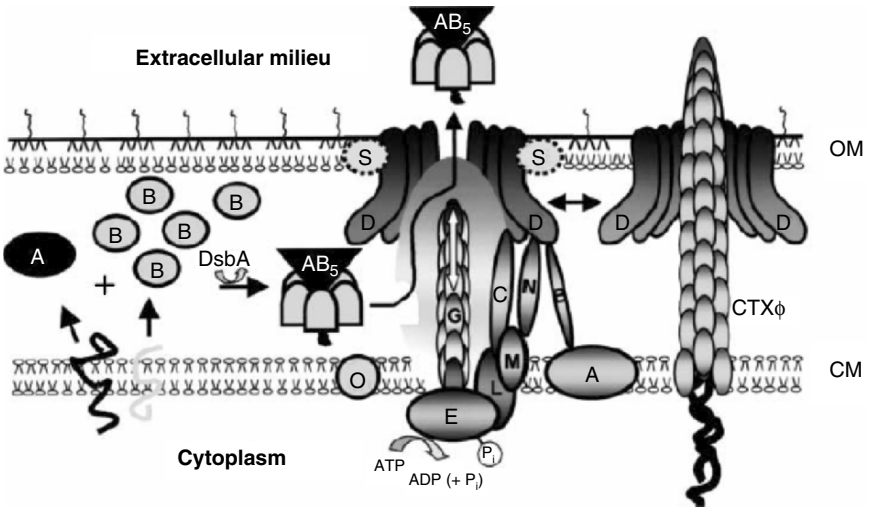

FIGURE 7. The type II secretion pathway (as used to secrete an A-B toxin). The toxin subunits (denoted "A" and "B") are secreted to the periplasm via the Sec system. In this location, the subunits fold and assemble into the AB5 holotoxin (with the assistance of the DsbA protein in some cases). The type II secretion machine recognizes a signal in the B5 subunit complex and engages the holotoxin for export. The holotoxin is targeted to the outer membrane channel and is released to the extracellular milieu. Protein D is a member of the secretin family and forms a pore in the outer membrane with the assistance of a lipoprotein (protein S). Protein G is the major subunit of a pilus-like structure that is hypothesized to act as a piston that may "push" the holotoxin through the protein D pore. Proteins E, L, M, and C are thought to facilitate communication between the subunits of the apparatus via ATP hydrolysis or phosphorylation. Proteins A, B, and N may function to stabilize the apparatus in certain species. Proteins H, I, J, and K (not shown) are likely processed by the protein O peptidase and expected to be part of the pilus-like structure with protein G. The protein D pore also functions in the extrusion of filamentous phage in some species and can shuttle between these two pathways (represented by the dark double-headed arrow) or may be in excess and available for both processes simultaneously. (From Sandkvist, 2001a.)

3.4.1. The Type II Protein D Component Forms the Outer-Membrane Pore and Is a Member of a Large Family of Outer-Membrane Proteins Called "Secretins"

The secretin family of outer-membrane proteins (OMPs) is composed of homologous proteins required for export processes such as type II and III secretion, type IV pilus biogenesis, and filamentous phage extrusion (Peabody et al., 2003; Sandkvist, 2001a; Yen et al., 2002). These proteins form oligomeric complexes that are extremely stable and form striking supramolecular, ringlike structures that can be observed with electron micrscopy (Bitter et al., 1998; Collins et al., 2001; Linderoth et al., 1997; Nouwen et al., 1999). The C-terminal domains of these family members are conserved, while the N-terminal domains are variable. It is thought that the secretins insert into the outer membrane using a conservered mechanism via the C-terminal domain, and then interact with specific substrates using the N-terminal domain. For some type II secretion systems (T2SS), a separate outer-membrane lipoprotein (protein S) is required for secretin protein D insertion and stabilization. The protein D pore is also used for extrusion of filamentous phages in some species, such as the *V. cholera* CTXϕ. This highlights the capacity of the secretins to be versatile portals for outer-membrane traversal.

3.4.2. The Type II Secretion Pilus/Piston Complex Is a Multiprotein, Membrane-spanning Structure

The protein G component is able to form long pilus-like structures (if overexpressed), and these structures are thought to span the cell envelope and may be used to drive the type II secretion process (Sauvonnet et al., 2000). The pilus structure is most likely associated with other type II components also. Experimental evidence supports the existence of a multiprotein complex consisting of proteins H, I, J, G, E, L, M, C, and D (Sandkvist, 2001a). Proteins A, B, and N are also thought to aid in the membrane-spanning organization of the type II apparatus. These proteins are a mixture of inner-membrane (proteins E, L, M, and A), outer-membrane (protein D), and envelope-spanning (proteins G, H, I, J, C) components. Protein E contains an ATP-binding site and may provide energy for secretion. Protein C has been hypothesized to transduce the energy from protein E to protein D in order to distribute power for the secretion process between individual components.

3.4.3. The Type II Secretion System and Type IV Pilus Biogenesis Apparatus Are Related

Several type II secretion proteins are homologous to proteins required for assembly of type IV pili involved in colonization and virulence of several pathogens. Some of these proteins (proteins G, H, I, J, and K) are homologous to the type IV prepilin subunits and are sometimes referred to as "pseudopilins" (Sandkvist, 2001a; Thanassi and Hultgren, 2000). The

pseudopilins use a membrane-bound peptidase for processing that can also be used by the type IV pilus subunits. The protein D secretin, as mention above, is utilized by both systems, and a pilus structure can be formed by system components in both cases. Details about type IV pilus biogenesis can be found in Chapter 7 on bacterial adhesions.

4. Sec-independent Secretion Systems

Protein substrates that are exported using Sec-independent secretion systems cross the cell envelope (inner membrane, periplasm, and outer membrane) without using the Sec system. These secretion systems (type I and type III) form an envelope-spanning apparatus that moves proteins from the cytoplasm to the extracellular environment. In addition, there is a Sec-independent mechanism for moving substrates across the inner membrane from the cytoplasm to the periplasm (the twin-arginine pathway).

4.1. Type I Secretion: ABC Transporters

ATP-binding cassette (ABC) transporters form a large family of proteins, which are widely distributed throughout all types of eukaryotic and prokaryotic cells (Binet et al., 1997; Davidson and Chen, 2004; Fath and Kolter, 1993; Tomii and Kanehisa, 1998). These transporters are able to bind and hydrolyze ATP and utilize this energy to transport molecules across membranes. In some cases, the direction of ABC transport is from the extracellular environment to the inside of the cell. In other cases, ABC transport systems export proteins from the inside of the cell to the outside. The latter systems have been termed type I secretion systems (T1SSs) and will be focused on here. A variety of proteins with different functions (including toxins and proteases involved in virulence) are secreted by the type I mechanism (Binet et al., 1997; Davidson and Chen, 2004; Fath and Kolter, 1993; Young and Holland, 1999). The prototypical type I transport system (and first-discovered ABC exporter in prokaryotes) is the *E. coli* alpha-hemolysin system (HylB, HylD, and TolC), though information has also been gained from studies of the enteric pathogen *S. marcescens* HasA and the plant pathogen *Erwinia chrysanthemi* PrtG systems (Binet et al., 1997; Fath and Kolter, 1993).

A diagram of type I secretion is given in Figure 8. The export apparatus consists of three proteins: an inner membrane ABC transporter (generically termed ABC), a protein that is anchored in the inner membrane and spans the periplasm (termed the membrane fusion protein, or MFP), and a pore-forming OMP (Thanassi and Hultgren, 2000; Young and Holland, 1999). The OMPs form complexes in the outer membrane that serve as a channel through this barrier. The MFPs are inserted in the inner membrane either by a hydrophobic amino terminus or by attachment of a lipid moiety to the N-terminus. The majority of the MFP protein extends outward from the inner

membrane, into the periplasm, and contacts the OMP channel and/or the outer membrane itself. The ABC proteins have both membrane-spanning domains and ATPase domains, and in some cases, these domains are actually two different proteins that function together as the ABC component. The structures of various ATPase domains (also referred to as nucleotide-binding domains, or NBDs) from several ABC proteins have been analyzed and reveal a two-arm structure (Schmitt et al., 2003). There is evidence that one

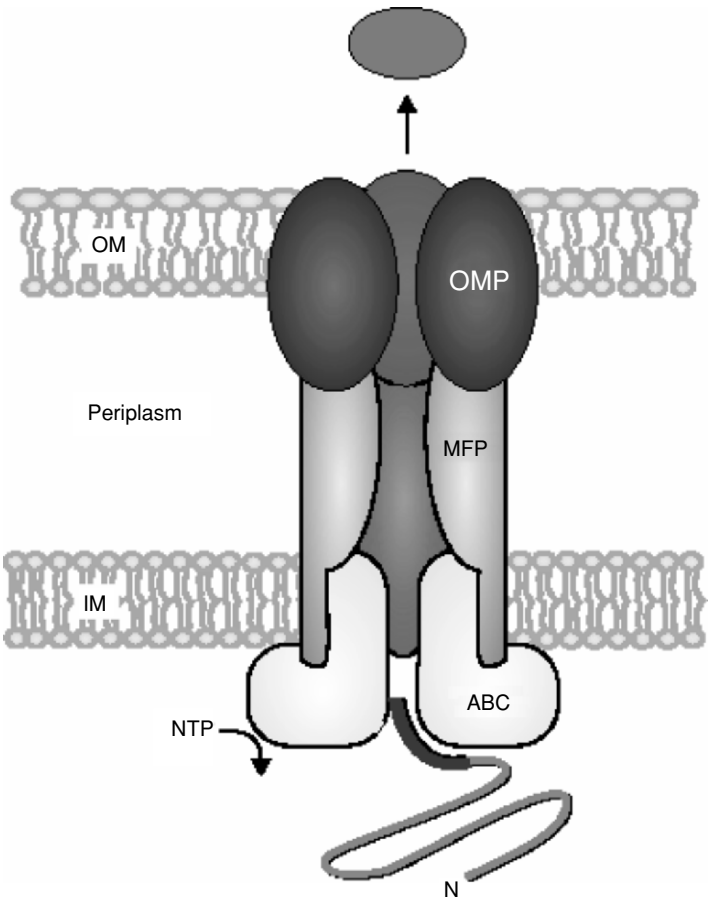

FIGURE 8. Model of type I secretion (ABC transporters). The outer memberane protein (OMP), membrane fusion protein (MFP), and ATP-binding cassette (ABC) protein are depicted in a complex that spans the Gram-negative cell envelope. The secreted substrate contains a Sec-independent C-terminal signal sequence, which is not cleaved upon export. ATPase activity from the ATP protein serves to drive the secretion process. The type I secretion complex allows Sec-independent transversal of the Gram-negative cell envelope. (From Thanassi and Hultgren, 2000. Reprinted with permission from Elsevier.)

of the arms contains a variable domain that may endow functional specificity to a given system. The type I secretion signal sequence is located in a 60 amino acid C-terminal domain of the protein substrate. There is little primary sequence homology between domains of different secreted substrates, though some proteins contain glycine-rich repeats (GGXGXD) that are involved in their export (Binet et al., 1997).

In addition to being utilized in Gram-negative bacteria, the ABC transporters function as a Sec-independent mechanism for protein export across the plasma membrane in Gram-positive bacteria (Tjalsma et al., 2004; Young and Holland, 1999). Due to the conserved nature of the ABC transporter family members, the organization of the proteins that constitute the transporter is very likely to be similiar between the Gram-negative and Gram-positive groups (except for the absence of the OMP component found in Gram-negative bacteria). However, some mechanistic differences may exist between Gram-positive and Gram-negative ABC systems. It has also been proposed that specific proteins containing a pseudopilin-like signal sequence are processed by an additional Sec-independent pathway in *B. subtilis*, though this pathway is not discussed here (Tjalsma et al., 2004).

4.2. Type III Secretion Systems

Type III secretion systems (T3SS) are widely distributed among Gram-negative animal and plant pathogens that establish an intimate interaction with eukaryotic host cells (Cornelis and Van Gijsegem, 2000; Galan and Collmer, 1999; Hueck, 1998). These systems are capable of translocating a protein (commonly called an "effector protein") from the inside of the bacterial cell to the inside of the eukaryotic host cell. The bacterial effector proteins are able to direct changes in host cell signaling pathways that engage the host in the lifecycle of the bacteria and facilitate propagation of the bacteria in the host. The discovery of type III secretion pathways has greatly increased our knowledge of how bacteria interact with host cells and is certainly one of the most important advances in the field of pathogenesis to date.

The secreted substrates of type III systems do not use the Sec system and are exported via the type III secretion apparatus. This apparatus can secrete proteins into the extracellular environment in addition to directing their translocation into host cells. Figure 9 provides a diagram of the type III secretion pathway. The type III apparatus consists of inner and outer membrane proteins, proteins that span the periplasm, and components that extend outward from the cell surface as a needle-like structure (Galan and Collmer, 1999). Table 1 provides a list of homologous type III system protein components that are present in *Salmonella enterica* SPI-1 and SPI-2 (*Salmonella* Pathogenicity Islands 1 and 2, respectively), *Yersinia* spp., *Shigella flexneri*, Pseudomonas aeruginosa, enteropathogenic *E. coli* and the *S. enterica* flagellar systems. The flagellar assembly system is an example of a T3SS that does not serve to translocate proteins into host cells, but rather forms an extracellular bacterial organelle. It has been frequently proposed that the flagellar

system is a common ancestor from which other T3SSs evolved; however, rigorous phylogenetic analysis refutes this hypothesis and suggests that they evolved independently of each other from a more ancient common ancestor (Gophna et al., 2003).

4.2.1. The Type III Secretion Basal Apparatus (the Inner- and Outer-Membrane Rings)

The outer-membrane component of the type III apparatus is a member of the secretin family of proteins, which form outer-membrane rings to allow passage through this membrane (Table 1, *Salmonella typhimurium* InvG

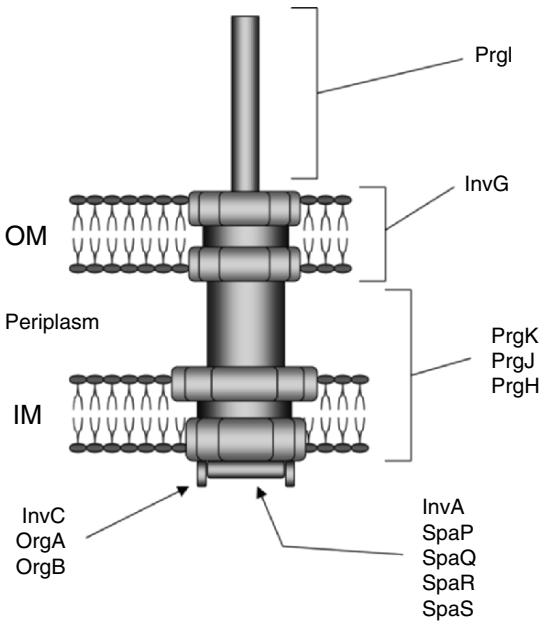

FIGURE 9. The type III secretion apparatus (the needle complex). The structural organization of the type III secretion apparatus in the Gram-negative cell envelope is depicted using the nomenclature from the *Salmonella typhimurium* pathogenicity island 1 (SPI-1) system. The needle is composed of PrgI protein subunits, which are secreted by the type III system and polymerize into the needle at the surface. The outer-membrane rings are formed by the InvG secretin protein. The inner-membrane rings (and most likely the periplasmic structure) are formed by the PrgH and PrgK proteins. The PrgJ protein is also a component of this substructure, though it is not essential for its formation. The InvA, SpaP, SpaQ, SpaR, and SpaS proteins are all predicted to be integral inner-membrane proteins and are likely to be associated with the base of the needle complex. It is thought that these proteins form the core of the export apparatus that is "housed" by the PrgH-PrgK needle base. The InvC protein is an ATPase whose activity is essential for type III secretion. Other proteins, OrgA and OrgB, are thought to associate with InvC at the cytoplasmic face of the needle complex.

homologs). These proteins are homologous to the type II secretion (and other) secretin proteins. The InvH homologs are outer-membrane lipoproteins that are thought to aid in the assembly of the secretin ring. The inner-membrane rings and periplasmic spanning structure are formed by the PrgH and PrgK group of homologs (Table 1). Both the secretin outer-membrane ring and inner-membrane PrgH/PrgK rings can form in the absence of each other using Sec-dependent secretion signals (Kimbrough and Miller, 2000; Sukhan et al., 2001). These components then come together to form the full basal ring structure. Different measurements of the rings formed by the

TABLE 1. Broadly conserved components of type III secretion systems.

Functional component	*Yersinia pestis*	*Salmonella typhimurium* SPI-1	*Salmonella typhimurium* SPI-2	*Shigella flexneri*	Flagellar system
Outer-memberane secretin	YscC	InvG	SpiA (SsaC)	MxiD	
Outer-membrane lipoprotein	YscW	InvH		MxiM	
Inner-membrane needle base component	YscD	PrgH		MxiG	FliG
Inner/outer-membrane needle base component	YscJ	PrgK	SsaJ	MxiJ	FliF
Needle base component	YscI	PrgJ	SsaI	MxiI	
Needle subunit	YscF	PrgI	SsaH	MxiH	FliC
Inner-membrane secretion apparatus	YscQ	SpaO	SsaQ	SpaO (Spa33)	FliN
Inner-membrane secretion apparatus	YscR	SpaP	SsaR	SpaP (Spa24)	FliP
Inner-membrane secretion apparatus	YscS	SpaQ	SsaS	SpaQ (Spa9)	FliQ
Inner-membrane secretion apparatus	YscT	SpaR	SsaT	SpaR (Spa29)	FliR
Inner-membrane secretion apparatus	YscU	SpaS	SsaU	SpaS (Spa40)	FlhB
Inner-membrane secretion apparatus	YscV	InvA	SsaV	MxiA	FlhA
Cytoplasmic	YscK	OrgA			
Cytoplasmic	YscL	OrgB	SsaK	MxiN	FliH
Cytoplasmic ATPase	YscN	InvC	SsaN	SpaL (Spa47)	FliI
Required for secretion	YscO	InvI	SsaO	SpaM (Spa13)	
Secreted regulator of needle length	YscP	InvJ	SsaP	SpaN (Spa32)	FliK

The proteins listed in each row are homologues and/or fulfill a common function during type III secretion.

InvG, PrgH, and PrgK are 21–40 nm in diameter and 9–20 nm in height with an inner core of 12 nm in diameter (Blocker et al., 2001; Kimbrough and Miller, 2000; Kubori et al., 1998).

4.2.2. The Proteins that Drive Type III Export Form a Complex on the Cytoplasmic Face of the Inner-Membrane Ring

There are six inner-membrane-associated type III secretion proteins that are highly homologous between type III systems of different species and are represented by the *S. typhimurium* InvA, InvC, SpaP, SpaQ, SpaR, and SpaS homologs (Galan and Collmer, 1999). The InvA protein is a large, polytopic inner-membrane protein that is one of the most conserved type III proteins, so much so that its DNA sequence is frequently used to identify homologous T3SSs in uncharacterized species. The SpaP-S proteins are smaller inner-membrane proteins that are also highly conserved. The InvC protein shares sequence similiarity with the alpha and beta subunits of the F1 component of bacterial F0F1 ATPases and has been shown to hydrolyze ATP in vitro. The InvC component is thought to be a soluble protein that associates with the type III machinery at the cytoplasmic face. Together, these proteins (possibly with other structural components) are thought to form the core of the type III export apparatus that serves to recognize the secreted substrates and drive their export across the cell envelope. The type III basal apparatus inner-membrane rings can be thought of as a structure that houses the export apparatus core. In the flagellar type III system, the core apparatus can be visualized as a supramolecular complex or "patch" that is present on the cytoplasmic face of the inner-membrane rings (striking electron micrographs of this patch can be seen in Katayama et al., 1996).

4.2.3. The Type III Secretion Needle

After the basal apparatus and export complex join together, the type III secretion needle can then be formed. Strains with mutations in the export complex components form an intact basal apparatus (i.e., the inner- and outer-membrane rings), but no needle structures. Thus, functional type III secretion is required for the needle to be formed. The needle is a multimeric structure composed of subunits belonging to the PrgI family of homologs (Table 1) and is approximately 45–80 nm long and 6–8 nm wide (Blocker et al., 2001; Hoiczyk and Blobel, 2001; Kubori et al., 1998). Complete needle complex structures (composed of the basal apparatus rings and the intact needle) have been purified from a number of bacterial species (*S. typhimurium*, *Shigella*, enteropathogenic *E. coli*, and *Yersinia*) and visualized by electron microscopy (Blocker et al., 2001; Hoiczyk and Blobel, 2001; Kubori et al., 1998; Sekiya et al., 2001). A 2–3 nm-wide hollow space (or "canal") in the center of the needle structure that extends from the basal apparatus through the needle is thought to be the conduit through which type III substrates pass upon export. The needles of the *Yersinia* system have been observed by

electron microscopy to actually puncture the host membrane surface and extend into the host cell. An exported substrate of the enteropathogenic *E. coli* (EPEC) type III system, EspA, can be visualized (using immunoelectron microscopy) extending from the tip of the needle structure in purified EPEC needle complexes. Multiple EspA molecules polymerize at the needle tip to form a long filament that interacts with the host cell surface and most likely allows translocation of effector proteins into the targeted host cell. The HrpZ protein, another substrate of the EPEC T3SS, can also be observed using the same techniques to be associated with the EspA filament (Li et al., 2002).

The length of the needle is regulated and can be increased by overexpressing the needle subunit protein inside of the bacterial cell (Kubori et al., 2000; Tamano et al., 2000). In the *S. typhimurium* SPI-1 type III system, the InvJ protein serves as a regulator that controls the length of the needle and the timing of substrate secretion. An *S. typhimurium* strain with an *invJ* mutation forms abnormally long needles and does not secrete any other type III substrates. A similar phenotype is also observed in a *spa32* mutant of *S. flexneri* and *yscP* mutant of *Yersinia* spp. (the Spa32 and YscP proteins are InvJ homologs) (Journet et al., 2003; Magdalena et al., 2002; Tamano et al., 2002). A further discussion on the regulation of secretion of type III substrates is given below.

4.2.4. Signals that Target Proteins for Type III Secretion: Lack of a Conserved Protein Signal Motif

Experimental evidence indicates that there are different molecular signals (or "tags") that specifically target substrates for type III secretion (Feldman and Cornelis, 2003; Lloyd et al., 2002; Ramamurthi and Schneewind, 2003; Russmann et al., 2002). Initial observations indicated that type III substrates are recognized by a noncleavable N-terminal protein motif. The first 15–17 amino acids of several type III substrate proteins can be fused to heterologous, nonsecreted reporter substrates to allow type III-dependent secretion of these reporters. However, there is no conserved N-terminal peptide sequence among different type III substrates. In addition, systematic mutagenesis of the N-terminal signals of type III substrates gave unexpected results. Frameshift mutations that completely altered the N-terminal peptide sequence of certain type III substrates did not prevent the protein from being recognized and secreted by the type III machinery. This led to a hypothesis that the 5′ end of the mRNA, rather than the N-terminal peptide sequence, is the signal for type III secretion. For some type III substrates, deletion of the N-terminus does not abolish its secretion and recognition of the substrate can still occur via a chaperone-mediated mechanism. Subsequent studies involving the manipulation and mutagenesis of several different type III substrates from different bacterial species indicate that the type III secretion signal does not conform to a strict paradigm and involves various factors.

There are three possible mechanisms for type III secretion substrate targeting that are supported by the experimental evidence: (1) an N-terminal peptide motif that consists of unordered (or unfolded) amino acids or possibly an alpha helical structure for some substrates; (2) an mRNA signal at the 5′ end of the transcript; and (3) binding of a chaperone protein and subsequent chaperone-mediated targeting to the type III apparatus. The involvement of chaperones in the secretion and function of type III substrates is discussed below.

4.2.5. Type III Secretion Chaperones

Chaperone proteins that interact with type III secretion substrates are typically small (about 15 kDa), acidic proteins (Cornelis and Van Gijsegem, 2000; Feldman and Cornelis, 2003). The absence of a particular chaperone generally results in the loss of secretion of its cognate substrate while not affecting the secretion of other substrates. The chaperones typically function as dimers and recognize specific domains in the cognate substrate protein, and this is usually in the N-terminal portion of the substrate for virulence-related systems. Although the type III chaperones share little amino acid sequence homology, they are remarkably similar in their tertiary structure as determined by crystallographic studies. The type III chaperones can be divided into two main categories (named after archetypical representatives from the *Yersinia* type III system): (1) the SycE family, which binds specifically to a single cognate substrate; and (2) the SycD family, which prevents premature association of two target proteins in the cytosol. A third category of type III chaperones has been proposed to exist with the discovery that the Spa15 protein of the *Shigella* type III system binds to three different target proteins (Page et al., 2002).

Type III secretion chaperones can function in several ways to facilitate proper export of substrates: (1) as stabilizing factors to prevent degradation of target proteins; (2) as antiaggregation factors to prevent the formation of nonfunctional protein complexes; (3) to guide substrates to the type III apparatus for secretion; (4) to determine the hierarchy of secretion of type III substrates; and (5) to prevent premature folding of substrates and hold them in a "secretion-competent" state.

4.2.6. Regulation of Type III Secretion

Type III secretion is regulated at several different levels to ensure the proper timing and order of type III secretion gene expression and substrate export. Below are some examples of: (1) type III gene transcriptional regulation and (2) ordered control of type III substrate export.

4.2.6.1. Transcriptional Regulation

The transcription of type III secretion genes is controlled by protein factors that respond to different environmental cues, and there is often a hierarchial

temporal order to the transcription of different groups of type III genes within the same system. One mechanism to achieve this transcriptional order is the retention of a negative regulatory factor in the bacterial cytosol until its secretion through the type III apparatus at a specific time (in the flagellar type III gene expression cascade). Upon secretion of the negative factor, transcription of the next subclass of type III genes is able to commence. Other types of regulatory cascades are described as well.

The Salmonella *PhoP-PhoQ System and the HilA-InvF Cascade.* The PhoQ membrane sensor protein and the PhoP response regulator form a conserved two-component system that regulates a variety of genes in response to changes in extracellular Mg^{2+} and Ca^{2+} concentration (Groisman, 2001). Bacterial growth in low levels (micromolar) of Mg^{2+} or Ca^{2+} promotes increased expression of PhoP-activated genes, whereas high levels (millimolar) of these ions represses expression of these genes. In *S. enterica*, the PhoP/PhoQ system negatively regulates the transcription of several SPI-1 type III secretion genes (most likely by activating a repressor of type III gene expression) (Lostroh and Lee, 2001). It is thought that when *Salmonella* senses low Mg^{2+} or Ca^{2+} concentrations inside host cells, the SPI-1 genes are repressed in a PhoP/PhoQ-dependent manner. Since the SPI-1 genes are used for invasion of host cells, it is likely that downregulation of these genes is advantageous inside the host cell after invasion is successfully completed. One of the SPI-1 genes that is negatively regulated by the PhoP/PhoQ system is *hilA*, which encodes a transcriptional activator of type III apparatus genes. HilA also activates the *invF* gene, the product of which serves as a transcriptional regulator of several effector proteins of the SPI-1 type III system. This cascade makes sense since HilA, an activator of the genes that build the type III apparatus, acts upstream of InvF, an activator of genes that encode substrates of that apparatus. A number of other regulators also play a role in controlling *hilA* expression including OmpR/EnvZ, SirA/BarA, RtsA, HilC, HilD, and HilE (Baxter et al., 2003; Ellermeier and Slauch, 2003). The use of multiple regulators underscores the apparent importance of "fine-tuning" *hilA* (and subsequent SPI-1 type III gene) expression.

The Shigella *VirF-VirB Cascade.* In *S. flexneri*, the AraC-like transcription factor VirF activates the expression of another transcriptional regulator, VirB, which in turn induces expression of the type III apparatus genes (Beloin and Dorman, 2003). The small nucleoid protein H-NS represses transcription of VirF, VirB, and the type III apparatus genes. When the growth temperature is raised to 37˚C (like that encountered during infection of the mammalian host), repression of VirF by H-NS is alleviated and the levels of VirF protein rise. VirF then serves to induce the expression of VirB, and VirB then displaces H-NS at the type III gene promoters to induce their transcription.

The Yersinia enterocolitica *VirF and YmoA Proteins.* In a manner similar to the *Shigella* VirF/VirB and H-NS model above, the *Y. enterocolitica* VirF and

YmoA proteins serve to regulate the transcription of type III secretion genes (*yop* genes) (Hueck, 1998). VirF is a transcriptional activator of the *yop* genes, while YmoA is also a small nucleoid protein (similar to H-NS) that represses expression of the *virF* and *yop* genes. When the growth temperature is raised to 37˚C, YmoA-mediated repression is relieved and cascaded expression of the *virF* and *yop* genes commences.

Enteropathogenic Escherichia coli *GadX and the* perABC *Operon.* EPEC use a T3SS to attach to, and efface the surface of, host intestinal cells. The genes encoding this T3SS are activated by the transcriptional regulators encoded in the *perABC* operon (Francis et al., 2002). In response to low pH (like that found in the stomach), the AraC-like transcriptional activator GadX represses *perABC* expression (and induces acid tolerance gene expression). In response to elevated pH (like that found in the intestinal compartment where EPEC would bind to host cells), GadX appears to act in an opposite manner to activate *perABC* expression (and decrease acid tolerance gene expression).

The Flagellar Type III Gene Expression Cascade. The transcriptional expression of the *S. enterica* flagellar genes occurs in distinct temporally regulated classes (Aldridge and Hughes, 2002; Chilcott and Hughes, 2000). The class 1 genes (or early genes) are *flhC* and *flhD*, which encode transcriptional regulators that form heteromultimeric complexes to direct sigma 70-dependent transcription of the class 2 genes (or middle genes). The class 2 genes encode components of the basal body/hook complex that form the membrane-bound base of the flagellum. Another class 2 gene is *fliA*, which encodes an alternative sigma factor, sigma 28, that directs transcription of the class 3 (or late) genes. However, the transcription of class 3 genes is strictly repressed until formation of the basal body/hook complex is complete, even though significant amounts of sigma 28 are present in the cytosol. This is because another class 2 gene product, FlgM, is an anti-sigma 28 factor that inhibits the activity of sigma 28. A switch in the flagellar type III secretion substrate specificity occurs upon completion of the basal body/hook complex that allows FlgM to be secreted, thus relieving the inhibition of sigma 28 and allowing class 3 transcription to commence. Interestingly, FlgM is also expressed as a class 3 gene, but the *flgM* class 3 transcript is targeted to the flagellar type III secretion apparatus to allow cotranslational secretion of FlgM. The cotranslational secretion of FlgM does not occur with the class 2 *flgM* transcript and is dependent on the FlgN chaperone protein.

4.2.6.2. Ordered Control of Type III Substrate Export

Certain type III system substrates are secreted before others, and this order is controlled by certain components of the secretion system. In addition, there are regulators that control the overall level of type III secretion.

The FliK/FlhB Substrate Specificity Switch. The FliK protein is a substrate for the *S. enterica* flagellar T3SS and belongs to a homolog family that

includes the *Salmonella* SPI-1 InvJ, *Shigella* Spa32, and *Yersinia* YscP proteins mentioned above that control needle length (Aldridge and Hughes, 2002; Chilcott and Hughes, 2000). This family of proteins functions to switch the specificity of the type III apparatus from substrates that form the needle structure (or hook component in the flagellar system) to substrates that serve as translocators and effectors (or flagellar subunits). Strains carrying mutations in this gene family form elongated needles (or hooks in the flagellar system, termed "polyhooks") and do not secrete other type III substrates. It is thought that after the needle component is exported and needle formation is completed, the FliK homolog is then secreted, and this signals the apparatus to switch specificity and secrete other substrates. The part of the flagellar type III apparatus that facilitates this switch is the FlhB protein, a member of a family that includes the *Salmonella* SPI-1 SpaS and *Yersinia* YscU proteins.

The Yersinia *LcrQ-YopH-YopE prioritization model.* In the *Yersinia* T3SS, there appears to be a preferential order (or "prioritization") to the secretion of the substrates LcrQ, YopH, and YopE (Feldman and Cornelis, 2003; Francis et al., 2002). The LcrQ protein has been implicated as playing a central role in regulating *Yersinia* type III gene expression and apparatus activity. This regulatory role for LcrQ is most likely related to its rapid secretion from *Yersina* cells upon induction of type III secretion. The complex of LcrQ and its chaperone SycH is thought to be targeted to the secretion apparatus first, resulting in preferential, rapid LcrQ export. Upon LcrQ secretion, cytoplasmic SycH is free to bind other substrates and aid in their secretion. One of these substrates is YopH, which is secreted after LcrQ. After YopH export, the complex of YopE and its chaperone SycE can bind the apparatus, and YopE is then secreted.

Salmonella *SPI-1 SipD and* Yersinia *YopN.* SipD and YopN are thought to act as "plugs" that control the overall amount of type III secretion (Hueck, 1998). Mutations in the genes encoding these products lead to increased secretion of numerous type III substrates without increasing the substrate expression or cytoplasmic levels.

4.2.7. Translocation of Type III Effector Proteins into Host Cells

A major step in type III secretion for bacterial pathogens is the translocation of effector proteins across the host cell membrane and into the host cell cytoplasm. T3SSs encode proteins, termed "translocators", that associate with targeted host cells and likely form a pore in the host cell membrane to allow the translocation of bacterial type III effectors into the targeted cell (Cornelis and Van Gijsegem, 2000; Hueck, 1998). The translocators for various type III systems are: (1) YopB, YopD, and LcrV for *Yersinia*; (2) SipB and SipC for *Salmonella* SPI-1; (3) IpaB and IpaC for *S. flexneri*; (4) EspB and EspD for EPEC; and (5) PopB, PopD, and PcrV for *P. aeruginosa. Yersinia, Shigella*, EPEC, and *P. aeruginosa* encode type

III systems that can actively lyse erythrocytes, and this activity is dependent on the genes encoding the translocators. Purified translocator proteins possess the ability to insert into erythrocyte or liposomal membranes, and a translocator-dependent change in membrane potential has been observed in artificial in vitro membrane systems. Moreover, the lysis of erythrocytes is not a general bursting open of the cell, but occurs through pores of a distinct size. This is based on the observation that molecules of large size are not released from the erythrocytes upon lysis and that a general release of intracellular protein from the erythrocytes is not observed. The hypothesized model for in vivo translocation is that the translocator proteins are secreted from the bacteria via the T3SS, and then somehow form multimeric structures in the targeted host cell membrane through which effector proteins enter the target cells. Three models as to how this could happen are: (1) the type III needle punctures the host cell membrane, the translocators are exported to the host cell cytoplasm, and a pore is formed to allow translocation of type III effectors; (2) the translocators are secreted into the extracellular milieu, they associate with the host cell membrane to form a pore, and the needle engages this pore to allow translocation of effectors; and (3) the translocators are secreted and associate with the tip of the needle, and the needle/translocator complex is inserted into the host cell membrane to allow translocation of effectors. Experimental evidence supports each of these models, but further study is needed to determine if any is actually correct.

4.2.8. Translocated Effectors of Type III Secretion Systems

Table 2 contains a list of effector proteins that are translocated into host cells by different type III systems to illustrate the number and function of effectors in different systems. Most of these effectors interact with host cell functions and alter host cell signaling pathways to facilitate an interaction between pathogen and host. Note that some of the proteins are multifunctional with roles in translocation and as effector proteins (SipB and SipC of *S. enterica* SPI-1; IpaB and IpaC of *S. flexneri*). A more extensive discussion of effector function during host cell invasion is given in Chapter 8.

4.3. The Twin-Arginine Pathway

4.3.1. The Twin-Arginine Pathway Exports Folded Cofactor-binding Proteins to the Periplasm or Inner Membrane

In addition to the Sec system, there is another protein export system that secretes proteins from the cytoplasm to the periplasm or inner membrane in Gram-negative bacteria, or to the extracellular milieu in Gram-positive bacteria. This system, which functions independently of the Sec apparatus, is termed the "twin-arginine translocation" (TAT) pathway due to the fact that the protein substrates secreted by this system contain a consensus N-terminal

TABLE 2. Effector proteins translocated into host cells by different type III systems.

***Yersinia* spp.**
YopE—GTPase-activating protein; cytoskeletal rearrangments
YopH—protein tyrosine phosphatase; disrupts peripheral focal complexes
YopM—interacts with two host cell kinases (PRK2 and RSK1); migrates to nucleus
YpkA—serine-threonine kinase; actin disruption
YopJ—cysteine protease and related to ubiquitin-like protein proteases; blocks MAPK and NFkappaB pathways
YopT—cysteine protease; cytoskeletal disruption
***Salmonella enterica* pathogenicity island 1 (SPI-1)**
AvrA—member of "avirulence" factor family that serves to limit the virulence of a pathogen; inhibits NF-kappa B pathway
SipA—binds actin; promotes formation of, and stabilizes, F-actin filaments
SipB—translocator; binds Caspase-1 host protein; induces cell death in macrophages and dendritic cells
SipC—translocator; binds actin; promotes formation of, and stabilizes, F-actin filaments
SopA—promotes induction of enteritis
SopB—inositol-phosphate phosphatase; stimulation of chloride secretion and cytoskeletal rearrangements
SopD—promotes induction of enteritis
SopE—GDP/GTP exchange factor; stimulation of actin cytoskeletal rearrangements
SptP—GTPase-activating protein; reverses effector-induced actin rearrangements
SspH1—leucine-rich repeat protein; inhibits NF-kappa B pathway signaling
SlrP—leucine-rich repeat protein; involved in colonization of murine Peyer's patches
***Salmonella enterica* pathogenicity island 2 (SPI-2)**
SpiC—binds Hook3 host protein; alters intracellular vesicular trafficking; possible translocator
SseF—modulates formation of host endosomal aggregates ("Sif" formations)
SseG—modulates formation of host endosomal aggregates ("Sif" formations)
SifA—required for Sif formations and *Salmonella*-containing vacuole (SCV) integrity
SifB—targeted to SCV and Sif formations
SspH1—leucine-rich repeat protein; inhibits NF-kappaB pathway signaling
SspH2—binds profilin host protein; localizes with polymerizing actin in host cells
SlrP—leucine-rich repeat protein
SseI—localizes with polymerizing actin in host cells
SseJ—predicted acyltransferase; localizes to SCV and Sif formations
PipB—targeted to SCV and Sif formations
SopD2—targeted to Sif formations and late endosomes
***Shigella* spp.**
IpaA—binds vinculin host protein; modulates cytoskeletal rearrangements
IpaB—translocator; binds Caspase-1 host protein; induces apoptosis of macrophages
IpaC—translocator; binds beta-Catenin and RanBMP host proteins; induces actin rearrangements
IpaH9.8—transported to host cell nucleus; inhibits NF-kappaB pathway
Pseudomonas aeruginosa
ExoS—ADP-ribosyltransferase; GTPase-activating protein; cytotoxin
ExoT—ADP-ribosyltransferase; GTPase-activating protein; cytotoxin
ExoU—phospholipase activity; cytotoxin; induces gene expression changes in host cells
ExoY—adenylate cyclase
Enteropathogenic *Escherichia coli*
Tir—inserted into host cell membrane; serves as receptor for bacterial adhesion; mediates cytoskeletal rearrangements involved with "pedestal" formation
Map—targeted to mitochondria and disrupts their function; induces filopodia formation
EspF—disrupts intestinal barrier function and induces host cell death
EspH—targeted to host cell membrane; modulates actin cytoskeletal rearrangments
Cif—blocks host cell cycle G2/M transition

signal peptide sequence with two conserved, consecutive arginine residues: SRRXFLK (where X is any residue) (Palmer and Berks, 2003). The TAT signal peptide is organized very similarly to the Sec signal sequence (with three subdomains); however, the TAT signal is typically longer than the Sec signal (in some cases, double the size) and is usually less hydrophobic and less positively charged. The major role of the TAT pathway is to export redox enzymes that are folded and bound to redox cofactors prior to export. This is in stark contrast to the Sec pathway, which secretes unfolded or partially folded substrates. Therefore, the mechanism of twin-arginine secretion is most likely very different from that of the Sec system and likely involves a usually large secretion pore. There is evidence that the TAT pathway can sense when a substate is properly folded and bound to a cofactor and will not engage a substrate that has not met these requirements. This has been termed a "folding quality control" mechanism (DeLisa et al., 2003).

In addition to redox proteins, the TAT pathway is also used to secrete virulence factors from *P. aeruginosa*, *E. coli* O157:H7, and *Agrobacterium tumefaciens* (Ding and Christie, 2003; Ochsner et al., 2002; Pradel et al., 2003). Strains of these bacteria that contain mutations in the *tat* genes are deficient for secretion of variety of substrates (including virulence factors) and are attenuated for virulence. In *P. aeruginosa*, proteins involved with phospholipase activity, iron acquisition, anaerobic growth, and certain catabolic functions contain the TAT signal sequence and depend on the TAT system for export. Moreover, a *P. aeruginosa tatC* mutant exhibits significantly reduced virulence in an animal pulmonary infection model. In *E. coli* O157:H7, the secretion of Shiga toxin 1 is severely decreased in a *tatABC* mutant and the verotoxicity of this mutant is significantly attenuated.

4.3.2. The Components of the TAT Transporter are TatA, B, C, and E

Four transmembrane proteins have been found to be involved with TAT-dependent export in *E. coli*: TatA, TatB, TatC, and TatE (Figure 10) (Palmer and Berks, 2003). A large operon encodes the *tatABCD* genes (mutation of *tatD* has no effect on TAT transport), while another separate gene encodes *tatE*. The TatA, TatB, and TatE proteins are sequence-related and are predicted to adopt a similar structure consisting of a transmembrane N-terminal alpha helix immediately followed by an amphipathic helix that associates with the cytoplasmic side of the inner membrane. The C-terminal domains of these proteins are variable in length and appear to be soluble. The TatA and TatE components are more related to each other than to TatB and have a partial functional redundancy: deletion of either *tatA* or *tatE* affects the secretion of certain substrates, but does not totally abolish TAT secretion (though a *tatA* deletion exhibits a more severe secretion defect). However, a *tatA tatE* double deletion completely inactivates TAT export. Deletion of *tatB* also inactivates TAT export and is not complemented by overexpression of *tatA* or *tatE*, indicating that TatA/TatE and TatB have functionally distinct roles. TatC is a

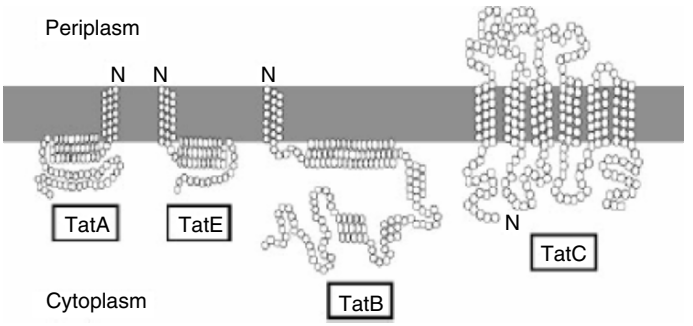

FIGURE 10. The components of the twin-arginine translocation (TAT) system. The TAT pathway is able to facilitate secretion of folded proteins including redox proteins and virulence factors. The proteins that form the TAT secretion apparatus are schematically depicted in their membrane conformations. The TatA, TatE, and TatB proteins are related in their primary sequences and are predicted to form similar structures consisting of a membrane-inserted N-terminal alpha helix followed by an amphipathic helix that associates with the cytoplasmic side of the inner membrane. The TatC protein is a polytopic membrane protein with six transmembrane helices. Purified TatA/TatB complexes have been shown to form ring-shaped structures that likely form a membrane pore. (From Palmer and Berks, 2003.)

highly hydrophobic, polytopic membrane protein with six transmembrane helices and is absolutely required for TAT secretion. The TatD protein is a DNase and appears to be nonessential for functional TAT export. The TAT transporter thus appears to be a complex consisting of TatA (or TatE), TatB, and TatC, where TatA is thought to form the main channel for substrate export and TatB and TatC function to recognize the secretion signals. TatA and TatB complexes can be isolated from *E. coli* cells overexpressing the *tatABCDE* genes and the purified complexes can be visualized by electron microscopy to form ring-shaped structures (Sargent et al., 2001). Interestingly, the central cavity of this ring structure is approximately 70 Å in diameter, a space predicted to be large enough to accommodate folded TAT substrates.

5. A Dual Sec-dependent and Sec-independent Secretion System: Type IV Secretion

5.1. Type IV Secretion Systems Are Related to DNA Conjugation Systems and Mediate the Transport of DNA and Proteins

T4SSs are used to transport DNA and protein macromolecules across membranes and can also mediate the translocation of these macromolecules into target cells (similar to type III systems) (Cascales and Christie, 2003; Christie,

2001; Ding et al., 2003; Yeo and Waksman, 2004). The type IV secretion family was initially established with the identification of homology between three macromolecular transport systems: (1) the *A. tumefaciens* T-DNA system, which moves oncogenic T-DNA into susceptible plant cells; (2) the conjugal transfer system of IncN plasmid pKM101; and (3) the transport system responsible for the secretion of the *B. pertussis* toxin, termed Ptl. The list of type IV secretion family members has grown considerably since this initial observation to include transport systems in *Legionella pneumophila*, *Helicobacter pylori*, *Brucella* spp., *Rickettsia prowazekii*, and *Neisseria gonorrheae*, as well as other conjugation systems including those of the IncP and IncF plasmid groups. The nomenclature of the T4SSs is based on the *Agrobacterium* T-DNA system, as this represents the prototypical (and historically best-known) type IV system. Although all the components listed below are named after the T-DNA system, the descriptions are representative of the other homologs in other systems (see Figure 11).

The type IV systems can be functionally grouped into three different categories: (1) conjugation systems that mediate transport of DNA between donor and recipient cells (*A. tumefaciens*, IncP, IncN, and IncF conjugation systems); (2) translocation systems that move effector proteins from the bacteria into host cells (*Legionella*, *Helicobacter*, *Bordetella*, and *Brucella* systems); and (3) DNA "uptake and release" systems that mediate exchange of naked DNA in the extracellular milieu (*Neisseria*, *Camphylobacter*, and *Helicobacter* systems) (Cascales and Christie, 2003). However, some systems can recognize both DNA and protein substrates, such as those encoded by *Agrobacterium* and *Legionella*. Along with T3SSs, the T4SSs represent a major mechanism by which pathogens can interact with host cells, and their discovery and characterization represents a significant step forward in our understanding of bacterial pathogenesis.

5.2. *The Type IV Secretion Apparatus Is a Multiprotein Complex that Spans the Bacterial Cell Envelope*

A diagram of the type IV secretion apparatus is given in Figure 12. This apparatus can be divided into two main components: (1) a large cell envelope–spanning complex that serves as the conduit for substrate export; and (2) a type IV "coupling protein" that serves to recognize and bring substrates to the large apparatus complex for export.

5.2.1. The VirB6–10 envelope-spanning complex

Experimental evidence indicates that the VirB6, B7, B8, B9, and B10 proteins (together with the VirD4-coupling protein and VirB4 and B11 ATPases discussed below) interact to form a conduit through which substrates pass to allow type IV secretion (Cascales and Christie, 2003; Ding et al., 2003;

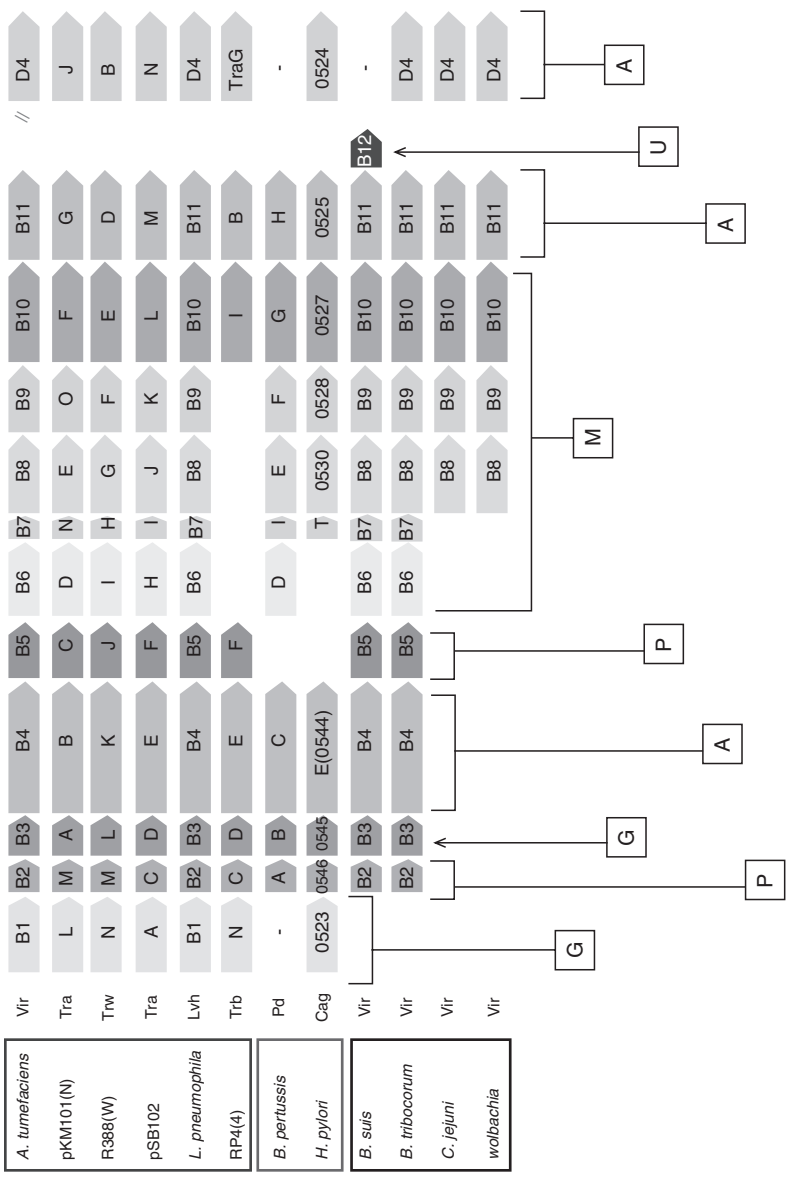

FIGURE 11. Gene nomenclature for different type IV secretion systems. The genes of different type IV secretion systems from a variety of species are aligned by common gene function. The functions of the genes are indicated by shades of the following colors and by the indicated lables: (1) ATPases—**A**; (2) membrane-embedded core structures—**M**; (3) pilus structures—**P**; (4) lytic transglycosylases—**G**; and (5) proteins with no assigned function—**U**. Further descriptions of these genes are in the text and in Figure 12. The names of the genes in the *Agrobacterium tumefaciens* system are commonly used to represent the type IV components in the standard nomenclature. (From Yeo and Waksman, 2004.)

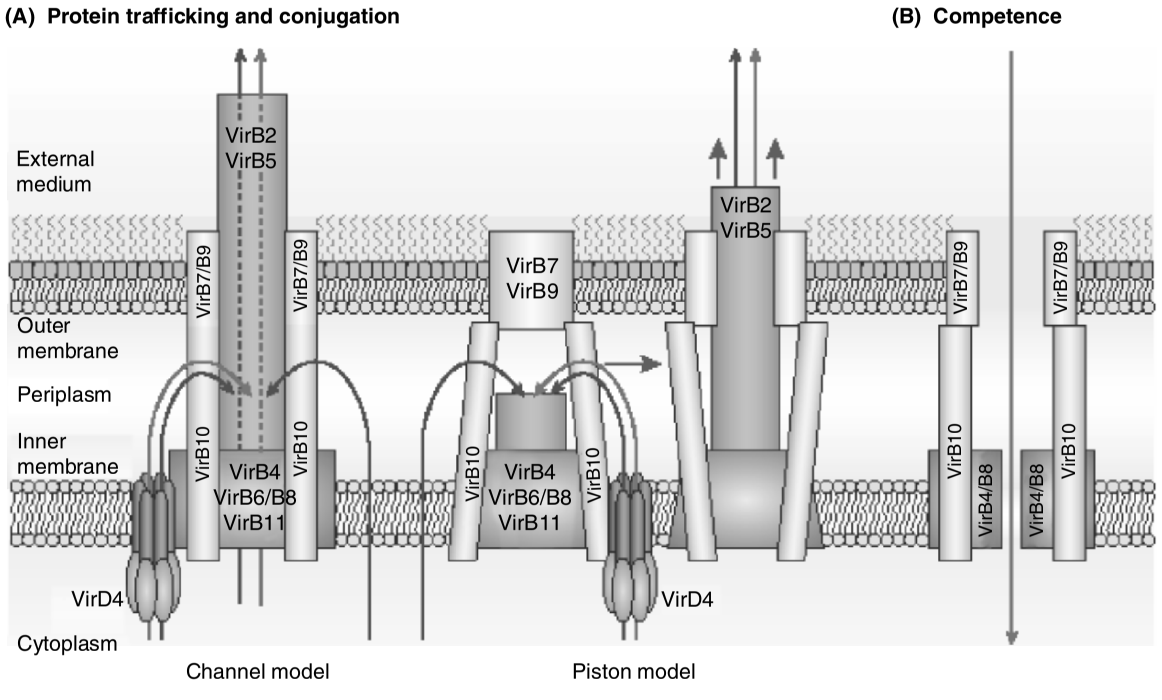

FIGURE 12. Models of type IV secretion mechanisms. A type IV secretion apparatus is schematically depicted in the Gram-negative cell envelope using the nomenclature from the *Agrobacterium tumefaciens* system. The VirB6, 7, 8, 9, and 10 proteins are thought to form a membrane-spanning structure. The VirB2 and VirB5 proteins are part of a pilus-like structure formed by the apparatus. The VirD4, VirB4, and VirB11 proteins are ATPases. The VirD4 protein forms a ringlike structure that serves as a critical component to "couple" the secreted substrate to the secretion apparatus. (A) Two models of type IV secretion are depicted with thin light gray arrows indicating DNA export routes and the dark gray arrows indicating protein export routes. In the channel model, the components of the type IV apparatus are predicted to form a channel through which substrates pass during transit to the extracellular milieu. In the piston model, substrates are loaded into the type IV apparatus and a piston-like structure formed by VirB2 and VirB5 "pushes" the substrate to the extracelluar milieu. Note that the dark gray arrows indicate that both Sec-dependent (through the membrane) and Sec-independent (through VirD4) routes can be used with the type IV apparatus. (B) Structure of the type IV DNA uptake system is depicted as a channel that allows the uptake of naked DNA from the environment. (From Cascales and Christie, 2003.)

Yeo and Waksman, 2004). The VirB6 protein is a highly hydrophobic, polytopic membrane protein that is proposed to form a channel in the inner membrane. The VirB9 protein, together with the lipoprotein VirB7, forms an outer-membrane complex that is critical in stabilizing the other apparatus components. VirB10 is a membrane protein with a short amino terminal cytoplasmic domain and a large carboxy terminal periplasmic domain that is proposed to link the inner- and outer-membrane portions of the apparatus. VirB8 is a membrane-localized protein that is essential for proper complex formation. Mutation of these proteins abolishes type IV transport and tends to disrupt the proper formation of the transport apparatus. The current model of the apparatus structure is one in which VirB6 forms an inner-membrane complex, VirB9 and B7 form an outer-membrane complex, and VirB8 and B10 function to bridge these two domains together.

5.2.2. The VirD4-coupling Protein

The VirD4 proteins belong to a family of putative ATPases and evidence suggests that they form a ringlike homohexamer structure with striking resemblance to the F1-ATPase and hexameric helicases (Cascales and Christie, 2003; Ding et al., 2003; Yeo and Waksman, 2004). The VirD4 component serves as the coupling protein that recognizes certain type IV substrates (both DNA and protein) and "hooks them up" to the type IV secretion apparatus. The use of the VirD4 component as a molecular adaptor is especially important during type IV conjugative DNA transfer since two important functions must be bridged during this process: (1) the mating pair formation apparatus, which mediates contact between donor and recipient cells and forms the pore through which the DNA gets transferred; and (2) a DNA-processing protein (termed a "relaxase"), which binds to, and cleaves, the target DNA that is to be transferred and serves to indicate the location where DNA transfer will commence. The VirD4 component forms a molecular bridge between the mating pair formation apparatus and the relaxase/DNA complex that allows the two functions to interact so that DNA transfer can proceed. An individual VirD4 component can recognize several different substrates. For example, the IncP plasmid VirD4 homolog, TraG, can recognize the relaxase complexes of different IncP and IncQ plasmids. However, it is important to note that not all substrates of T4SSs use the VirD4 component for recognition and export.

5.2.3. The VirB4 and VirB11 ATPases

The VirB4 and VirB11 components contain conserved Walker A box nucleotide-binding domains, display ATPase activity in vitro, and are thought to localize to the inner membrane to provide energy for type IV transport (Cascales and Christie, 2003; Ding et al., 2003; Yeo and Waksman, 2004). Members of the VirB11 family (TrbB of the IncP plasmids, TrwD of plasmid R388, and RP0525 from *H. pylori*) form higher-order, homomultimeric, ringlike structures

in vitro that can be observed via electron microscopy. For TrbB, homomultimer formation is dependent on the Walker A motif.

5.2.4. The VirB2 T-pilus

T4SSs (especially those involved with conjugation) often direct the formation of a pilus that could be used for several hypothesized functions: (1) as a conduit for transfer of DNA and protein substrates; (2) as a sensory receptor to receive host cell signals that initiate transfer; and (3) to mediate adhesion and close contact of the bacteria to host cells to facilitate type IV translocation (Lai and Kado, 2000). The *Agrobacterium* type IV pilus (termed the "T-pilus") is a multimeric structure made up of VirB2 subunits. The VirB2 polypeptide is processed from a 12.5 kDa proprotein to a 7 kDa mature subunit protein that is incorporated into the pilus. Remarkably, the 7 kDa form of VirB2 undergoes a cyclization reaction in which the amino and carboxy terminal ends of the protein are joined together in an intramolecular "head-to-tail" peptide bond. This cyclization reaction has also been shown to occur with the VirB2-homolog TrbC from IncP plasmid RP4. Large intracellular pools of cyclized VirB2 subunits accumulate in the cytoplasm, and upon an unknown signal from the host, the subunits are polymerized into an extracellular pilus by the type IV apparatus. Studies indicate that the T-pilus does not retract like other pili but instead winds into compact coils that presumedly bring the bacterium and host cell into close contact. The VirB5 protein appears to be a minor component of the T-pilus as it can be observed to be associated with the T-pilus and copurifies with polymerized VirB2 subunits.

Pertussis toxin export is an example of Sec-dependent type IV secretion. Most examples of type IV secretion (*Agrobacterium* T-DNA transfer, conjugative DNA transfer, and effector translocation into host cells) are considered to utilize a Sec-independent mechanism of type IV secretion. However, the export of pertussis toxin (PT) via the Ptl type IV system in *B. pertussis* is a notable example of Sec-dependent type IV secretion (Christie, 2001). The A and B subunits of PT are secreted from the cytoplasm to the periplasm by the Sec pathway, and they assemble in the holotoxin at this periplasmic location. The Ptl type IV system then recognizes the holotoxin and secretes it across the outer membrane.

6. Conclusions: Examples of Protein Secretion Systems in Different Pathogens

An essential requirement for pathogens to interact with host cells is the ability to move key virulence proteins from the intracellular cytosol to the extracelluar environment. As illustrated in this chapter, bacterial pathogens have evolved several different mechanisms for protein secretion and

translocation to the host cell. Indeed, it is very common for a given pathogen to use several of these pathways to secrete different virulence factors. Often, the host–pathogen interaction is a complex, multilayered process that involves the secretion of many bacterial proteins with different functions. Table 3 contains a list of the known protein secretion pathways described in this chapter and the pathogens that utilize each mechanism to secrete the indicated substrates. Although not intended to be an exhaustive list, it serves both as a summary of the information in this chapter and as a reference that illustrates the use of each pathway in an array of pathogens. A number of secretion systems have been shown to be present in several newly sequenced bacterial genomes, but not shown to be functionally active; these examples were not included. The list is a testament to the power of evolution that considerably different pathogens can utilize the same secretion mechanism to facilitate their own unique host–pathogen interaction.

Questions to Consider

1. At this point in our knowledge, Gram-negative bacterial cells seem to have evolved many more protein secretion mechanisms than Gram-positive cells. What is the most likely reason for this phenomenon?

The Gram-negative cell envelope, with its inner and outer membranes and periplasmic space, is an interface that presents more options for the evolution of different kinds of secretion systems than the somewhat simpler Gram-positive cell envelope. For example, not only can mechanisms evolve to move proteins across the inner membrane but there can also be evolution of outer-membrane traversal mechanisms. In addition, mechanisms have evolved to move proteins across both membranes in a single step. In Gram-positive cells, there is a single membrane separating the cytosol from the extracellular environment. The presently characterized secretion systems in Gram-positive cells are also found in Gram-negative cells. The secretion systems unique to Gram-negative cells are used for secretion across the outer membrane.

2. What is a "secretin" and what protein secretion systems utilize them?

Secretins are proteins that form a channel or pore in the outer membrane of Gram-negative cells to allow the movement of proteins across this membrane. The different secretin proteins are homologous and form a family of proteins. Type II and type III secretion systems utilize secretins as essential components.

3. In the *Salmonella typhimurium* type III secretion system, the inner- and outer-membrane rings (formed by the PrgH/PrgK and InvG proteins, respectively) are formed even in strains containing mutations in the genes encoding components of the export machinery such as *invA* and *invC*. Therefore, is the type III secretion mechanism required for assembly of the basal rings? How do

TABLE 3. Examples of protein secretion systems in different pathogens (functional class of substrates is underlined, followed by example pathogens and specific proteins or systems).

Sec-dependent secretion systems
1. Type V (autotransporters)
IgA1 protease
Neisseria spp. IgA1 protease
Haemophilus spp. IgA1 protease
Serine protease autotransporters from Enterobacteriaceae (SPATEs)
Escherichia coli EspP, Pet, and Tsh
Shigella flexneri SepA, Pic, and SigA
Other proteases
Pseudomonas fluorescens PspA, PspB
Pasteurella haemolytica Ssa1
Serratia marcesens PrtS, PrtT, Ssp-H1, and Ssp-H2
Adhesins
Bordetella spp. Pertactin, TcfA, and Vag8
Escherichia coli AIDA-I, TibA, and Ag43
Haemophilus influenza Hap, Hia, and Hsf
Helicobacter pylori BabA
Moraxella catarrhalis UspA1 and UspA2h
Mediator of intracellular motility
Shigella flexneri IcsA
2. Two-partner secretion (TPS)
Filamentous hemagglutinin
Bordetella pertussis FHA
Calcium-independent hemolysin
Serratia marcesens ShlA
Proteus mirabilis hemolysin
Adhesins
Haemophilus influenzae HMW1, HMW2, and HxuA
Other TPS substrates
Edwardsiella tarda EthA
Haemophilus ducreyi LspA1 and LspA2
3. Chaperone/usher
Pilus/fimbrial assembly
Escherichia coli type 1 pili, P pili, and F17-G fimbriae
Pseudomonas aeruginosa CupA, CupB, and CupC fimbrial systems
Acinetobacter baumannii csu fimbrial system
Yersinia pestis F1 capsular antigen
4. Type II (main terminal branch)
Variety of substrates in many pathogens
Pseudomonas aeruginosa exotoxin A, elastase, LasA, LipA, LipC, phospholipase C
Vibrio cholera cholera toxin (CT), HAP, chitinase, neuramidase, lipase
Klebsiella oxytoca pullulanase
Aeromonas hydrophila aerolysin, amylase, phospholipase, serine protease, DNAse
Burkholderia pseudomallei lipase, protease, phospholipase C
Legionella pneumophila acid phosphatase, lipase, phospholipase A, RNAse, Msp protease
Sec-independent secretion systems
1. Type I (ABC transporters)
Hemolysins
Escherichia coli HlyA
Proteus vulgaris Hly system

(*Continued*)

TABLE 3. Examples of protein secretion systems in different pathogens (functional class of substrates is underlined, followed by example pathogens and specific proteins or systems)—Cont'd

Morganella morganii Hly system
Actinobacillus spp. hemolysins
Enterococcus faecalis hemolysin system
Variety of other type I protein substrates
Bordetella pertussis cyclolysin
Pseudomonas aeruginosa alkaline protease
Pasteurella haemolytica leukotoxin
Serratia marcescens Zn protease
Polysaccharide-containing substrates
Bacillus subtilis TagHG (polyglycerolphosphate)
Haemophilus influenzae BexABC (polyribosylribitol)
Klebsiella pneumoniae RfbBA (D-Galactan I)
Agrobacterium tumefaciens ChvA (beta-(1-2)-Glucans)
2. Type III
Variety of substrates in many pathogens
Yersinia spp. Ysc-Yop system
Salmonella enterica Salmonella pathogenecity island 1 (SPI-1)
Salmonella enterica Salmonella pathogenecity island 2 (SPI-2)
Salmonella enterica flagellar system
Shigella spp. Mxi-Spa-Ipa system
Enteropathogenic *Escherichia coli* (EPEC) Esp system
Pseudomonas aeruginosa Psc-Pop-Exo system
3. Twin-arginine translocation (TAT) transporters
Variety of substrates
Pseudomonas aeruginosa phospholipase C, iron acquisition proteins, catabolic proteins
Enterohemorragic *Escherichia coli* (EHEC) Stx1 Shiga-toxin
Agrobacterium tumefaciens TAT system
Dual Sec-dependent and Sec-independent secretion system
1. Type IV
Protein substrates
Bordetella pertussis Ptl system (Pertussis toxin) [Sec-dependent]
Legionella pneumophila Dot-Icm system
Helicobacter pylori Cag system
Brucella spp. VirB system
DNA substrates
Agrobacterium tumefaciens T-DNA system
Helicobacter pylori Com system
Neisseria gonorrhoeae DNA export system
IncF, IncI, IncP, and IncW plasmid DNA conjugation systems

you suppose the ring proteins are exported from the cytoplasm to their respective locations?

Since *invA* and *invC* mutations abolish function of the type III export apparatus, type III secretion is not required for formation of the basal ring structures. The ring proteins (PrgH, PrgK, and InvG) are exported from the cytoplasm to their membrane locations by the Sec system.

4. Secreted proteins carry signals that target them for transport via a certain secretion system. How would these signals be identified? Name three ways in which a researcher could identify the secretion signals on protein substrates.

Possible approaches to identify the secretion signal in a given substrate include:

(1) Perform a mutational analysis of the gene encoding the protein substrate to help localize the signal. For example, constructing N-terminal or C-terminal truncations would allow one to determine if these sections of the protein are essential for secretion, and sets of different size deletions would allow more precise identification of the signal.
(2) Fuse different sections of the substrate protein to a nonsecreted reporter protein (which is easily identified by Western blot or other means) to determine which section allows secretion of the reporter. For example, if you fused the N-terminal 80 amino acids of a secreted substrate to a reporter and observed reporter secretion, the secretion signal likely resides in this 80 amino acid domain.
(3) If a given secretion mechanism recognizes a number of substrates, one could align the protein sequences of the different substrates and look for common amino acid domains between the different proteins. Commonly, areas of homology between different substrates (especially at the N-terminal or C-terminal ends) will signify a secretion signal. However, this would have to be experimentally verified using one of the first two approaches mentioned above.

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Chapter 10

Toxins as Host Cell Modulators

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Historical Landmarks

1884 Recognition of the role of soluble factors involved in infectious disease—diphtheria toxin (Loeffler, 1884).

1890 Identification of tetanus neurotoxin as the sole cause of the disease, tetanus (Faber, 1890; Tizzoni and Cattani, 1890; von Behring and Kitasato, 1890).

1890 Use of chemically inactivated diphtheria toxin as a vaccine toxoid (von Behring and Kitasato, 1890); for this accomplishment, von Behring was later awarded the Nobel Prize in Physiology and Medicine in 1901.

1892 Recognition of the cell and tissue specificity of some toxins—neuroselective binding and spinal cord activity of tetanus toxin (Bruschettini, 1892).

1936 Recognition that toxin production by pathogenic bacteria is regulated—diphtheria toxin (Pappenheimer and Johnson, 1936).

1951 Recognition that some toxins may originate from mobile genetic elements—diphtheria toxin (Freeman, 1951).

1958 Identification of first toxin receptor—tetanus toxin association with host cells via gangliosides (Van Heyningen, 1958).

1967 First intracellular target identified—elongation factor-2 targeted by diphtheria toxin—confirms the notion that toxins could function within eukaryotic cells (Collier, 1967; Goor and Pappenheimer, 1967).

1969 First enzymatic activity associated with a toxin—ADP-ribosylation by diphtheria toxin—provides an explanation for why the cellular effects of just a few toxin molecules can be amplified so greatly (Gill et al., 1969; Honjo et al., 1969).

1978 First toxin gene cloned—heat-labile toxin—makes genetic manipulation of bacterial toxin possible (So et al., 1978).

1978 First magic bullet constructed—the diphtheria toxin catalytic domain was delivered to cells by heterogeneous cell recognition molecules—demonstrates that the potency of some toxins can be harvested for potential medical applications (Gilliland and Collier, 1980; Gilliland et al., 1978, 1980).

1986 First crystal structure—exotoxin A—ushered in the era investigating toxin structure–function relationships (Allured et al., 1986)

1989 First superantigen identified—staphylococcal enterotoxin B (White et al., 1989).

1990 First type III toxin effectors identified—*Yersinia* Yop proteins—confirms the idea that some bacterial toxin effectors can be directly injected into host cells (Michiels et al., 1990).

1993 First protein effector identified to be secreted by type IV secretion system—pertussis toxin (Covacci and Rappuoli, 1993; Weiss et al., 1993).

1. Introduction

To be successful, pathogenic bacteria sometimes must remodel host cells and tissues into suitable niches for colonization. Remodeling strategies include altering epithelial membrane barriers to provide optimal surface for bacterial attachment and/or invasion, as well as circumventing or subverting the host's immune response to facilitate persistence. Each of these strategies, as well as others described throughout this book, requires that pathogenic bacteria alter specific properties of host cells and tissues through the action of molecular effectors called toxins.

In this chapter, we shall explore the fascinating world of bacterial toxins. A prominent theme is that pathogenic bacteria produce toxins to exert modulatory effects on eukaryotic cells and tissues, often resulting in the remodeling of the host environment. Major topics to be covered will include the regulation, export, and intoxication mechanisms of bacterial toxins. Toxins will be grouped into classes according to whether their cytotoxic effects are elaborated inside or

outside of the eukaryotic cells they target. The biochemical function of toxins will be discussed in some detail as one of the better understood aspects of toxin action. We will consider reasons why uncovering the exact roles of these potent effectors in host–pathogen interactions can be a puzzling and problematic issue. Illustrations will be given of how the exploration of cellular intoxication mechanisms has stimulated breakthroughs in several other fields. Finally, we will spotlight recent advances in the development of effective countermeasures against the use of toxins and toxin-producing bacteria as bioweapons.

2. The Importance of Toxins in Bacterial Pathogenesis

Toxins are among the most important implements within the toolbox of virulence factors employed by pathogens to successfully colonize and persist within a host. In fact, toxins are responsible for one of the earliest notions of virulence—that pathogenic bacteria secrete factors that mediate all of the symptoms of disease. This is clearly the case for the *Clostridium botulinum* neurotoxins, which are released, ingested by the host, and are ultimately responsible for all the pathogenesis associated with botulism, even in the absence of infection. Other toxins as well cause most or all of the symptoms associated with disease, and/or are critical factors for colonization of the host. However, the advent of more sophisticated genetic approaches, as well as the recognition of the importance of the host and environmental factors in the disease outcome, has underscored the need to consider the complex and multifactorial nature of virulence. Because a given toxin may sometimes be elaborated as only one of many virulence factors by a pathogen, assessing the role of this single factor in disease is not always straightforward. The in vivo role of a toxin cannot always be readily quantified by simply "knocking out" the gene encoding the toxin, which is generally considered the gold standard for assessing the importance of specific factors in pathogenesis. In fact, identifying the exact contributions of toxins to the overall virulence strategies of pathogens can at times be more perplexing than understanding the molecular and cellular mechanisms underlying cytotoxicity. One possible reason for this is that toxins can often induce several quantifiable effects on different types of mammalian cells. Identifying the most relevant of these toxin-mediated effects to pathogenesis and disease can be difficult. Despite such challenges, the importance of toxins to bacterial pathogenesis has been recognized for over a century.

3. What Do Toxins Do? An Overview of Toxins at the Cellular and Molecular Level

Historically, the deployment of toxins by pathogenic bacteria has been considered a rather "heavy-handed" approach for dealing with the host. An assumption for many years was that the "job" of all toxins was to kill

eukaryotic cells (Figure 1). Although some toxins, such as diphtheria and Shiga toxins, ultimately do cause cell death, many toxins, in fact, do not kill, but instead act to modulate the properties of eukaryotic cells (Figure 1). For example, cholera toxin, secreted by the intestinal pathogen *Vibrio cholerae*, alters signal transduction in epithelial cells to induce changes in membrane channels, ultimately causing significant osmotic imbalances within the intestinal lumen. These modulatory effects of cholera toxin, which are not sufficient to kill intoxicated cells outright, are ultimately responsible for the onset of the massive diarrhea associated with cholera—which obviously can be devastating to the infected individual. This example underscores the importance of considering toxin action at the "global," or host level, in addition to the cellular level.

A brief overview here of bacterial toxins is important to provide the necessary framework for a more detailed discussion later of how these important virulence factors interact with host cells and modulate their functions. Brief

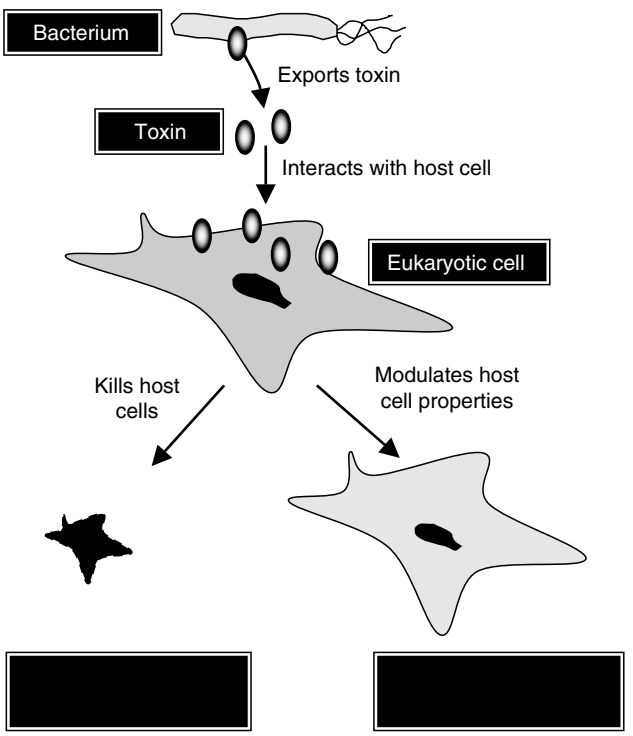

FIGURE 1. Cellular outcome of bacterial toxin action. Pathogenic bacteria synthesize and export protein bacterial toxins. The interactions of toxins with sensitive eukaryotic cells ultimately result in (1) cell death or (2) modulation of cellular properties in the absence of cell death.

overviews will be provided for (1) toxin classes and names; (2) overall cellular intoxication mechanisms; (3) eukaryotic targets; and (4) toxin structure and function.

3.1. An Overview of Toxin Classes and Names

As scientists, we need to make sense of the world about us in a systematic and orderly manner. However, one could argue that the naming of toxins has been neither systematic nor orderly, leaving us with a bewildering array of toxin names. Nonetheless, understanding the "language" used by toxin investigators is important for navigating this exciting and dynamic field (Table 1).

Protein toxins that are exported from bacterial cells into the extracellular environment have collectively been classified as exotoxins (Table 1). In contrast, the term "endotoxin" refers to the lipopolysaccharide-based outer leaflet of the Gram-negative outer membrane, which will not be discussed further within this chapter (see Chapter 6). We will also discuss toxins that are not exported into the extracellular environment, but are instead injected from the bacterium directly into the cytosol of the host cell. These toxins are not normally referred to as exotoxins, but are instead called "effectors" or "effector toxins." Some exotoxins are further classified as "enterotoxins," because they cause symptoms associated with enteric disease, such as diarrhea and vomiting. Exotoxins that are capable of intoxicating multiple cell types are sometimes referred to as "cytotoxins." Toxins can also be grouped according to target cell type or organ sensitivities. For example, neurotoxins, cardiotoxins, and hepatotoxins target cells of the nervous, cardiac, and hepatic systems, respectively.

TABLE 1. Classification and names of toxins.

Classification	**Description**
Cytotoxins	Exotoxins that are capable of intoxicating multiple cell types
Effectors or effector toxins	Protein toxins that are injected from the bacterium directly into the cytosol of the host cell
Endotoxins	Lipopolysaccharide-based outer leaflet of the Gram-negative outer membrane
Enterotoxins	Protein toxins that cause symptoms associated with enteric disease
Exotoxins	Protein toxins that are exported from bacterial cells into the extracellular environment
Naming system	**Examples**
Associated disease or organism	Diphtheria toxin, cholera toxin
Biochemical activity	Adenylate cyclase toxin
Gene products	Exotoxin A, exotoxin S, vacuolating cytotoxin
Symptoms associated with the action of the toxin	Lethal toxin and edema toxin

A number of the earliest-discovered toxins were named to indicate the associated disease or disease-causing organism (Table 1). Thus, remembering that diphtheria toxin causes the symptoms of diphtheria and cholera toxin is made by *V. cholerae* is relatively easy. Still other toxins were named for their biochemical activities (e.g., adenylate cyclase toxin from *Bordetella* spp.), or symptoms associated with the action of the toxin (e.g., lethal and edema toxins from *Bacillus anthracis*). Probably the most systematic approach—albeit often the least descriptive—has been the use of the standard designation for gene products, such as ExoA, ExoS, and VacA (e.g., exotoxin A and exotoxin S from *Pseudomonas aeruginosa*, and vacuolating cytotoxin from *Helicobacter pylori*, respectively). More recently, toxins have increasingly been named according to this last approach.

3.2. An Overview of Cellular Intoxication Mechanisms

Pathogenic bacteria synthesize and export protein toxins that act upon, or "intoxicate," eukaryotic target cells within the host. Exported toxins can act from either the outside or, alternatively, from the inside of target cells (Figure 2).

3.2.1. Extracellular-acting Toxins

Extracellular-acting toxins modulate target cells in three very different ways (Figure 2). Members of one group are enzymes that catalyze the breakdown of components at the plasma membrane (or sometimes the extracellular matrix). Members of the second group do not function as enzymes, but rather insert into the lipid bilayer of the plasma membrane to form pores or channels of various sizes. Despite differences in the ways these toxins interact with target cells, both groups of extracellular-acting toxins ultimately act to destabilize the plasma membrane, as well as cause other changes within the cell. An additional important mechanism employed by extracellular-acting toxins is to directly interact with, and bind to, plasma membrane receptors resulting in the activation of eukaryotic cellular signal transduction.

3.2.2. Intracellular-acting Toxins

Intracellular-acting toxins generally act upon and modify specific intracellular eukaryotic targets by enzymatic mechanisms (Figure 2). These toxins are potent, in part, because a single toxin molecule is capable of altering the structure and function of many target molecules, which in turn affects normal cell function. Intracellular-acting toxins attain access to their eukaryotic targets by two overall mechanisms. Some toxins are injected directly from the bacterial cell into the eukaryotic cell, while others are first exported into the extracellular host environment, and must then be transported into target cells in order to be effective.

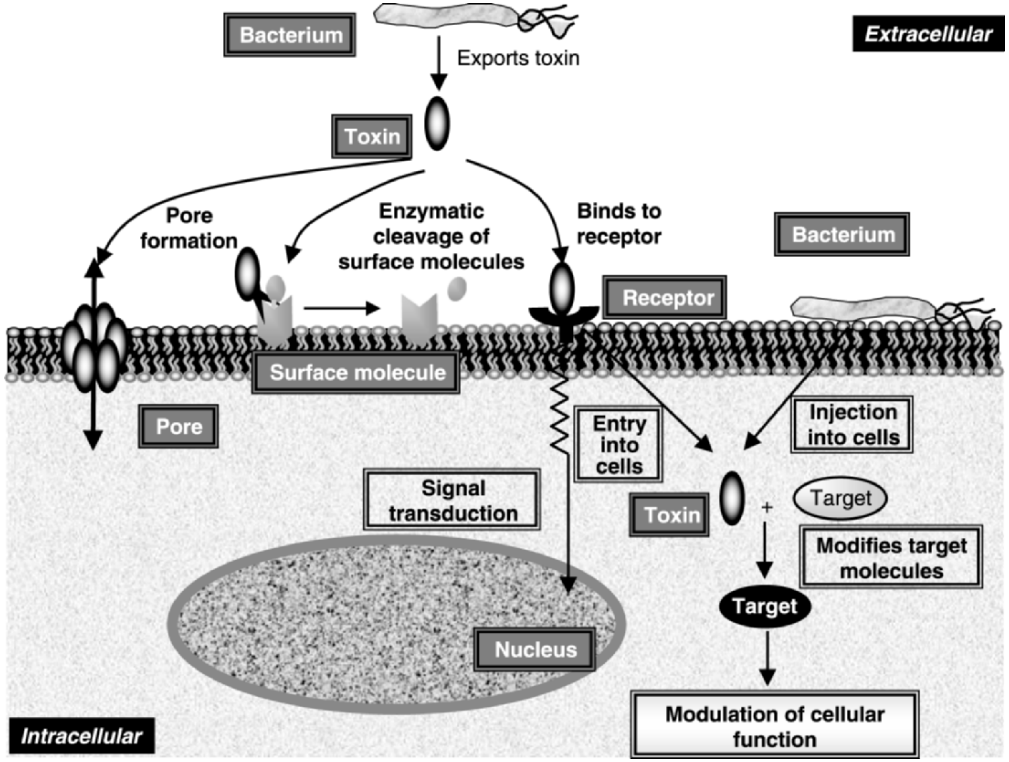

FIGURE 2. An overview of cellular intoxication mechanisms. Subsequent to export from pathogenic bacteria, toxins modulate eukaryotic cells by: (1) forming pores in the plasma membrane; (2) cleaving molecules off the plasma membrane; (3) binding to plasma membrane receptors to activate signal transduction; or (4) modifying intracellular targets in such a way as to alter their functions, which in turn, modulates one or more aspects of normal cellular function.

3.3. An Overview of Eukaryotic Targets

Bacterial toxins generally act upon specific structural or functional features of eukaryotic targets. In this section, we will briefly describe some common targets for bacterial toxins.

3.3.1. The Plasma Membrane as a Target

Many bacterial toxins act at the eukaryotic plasma membrane, which normally functions as an important barrier between the harsh extracellular environment and the complex matrix of organelles and molecular complexes inside the cell. Important cellular functions that are carried out or regulated at the plasma membrane surface can be altered by the action of bacterial toxins. Toxins that insert into the membrane to form channels or pores spanning the lipid bilayer modulate the permeability barrier, which sometimes, but not always, leads to cell death (e.g., aerolysin, α-hemolysin). Toxins that degrade proteins or lipids at the plasma membrane act to remodel the molecular composition of the membrane, and sometimes destabilize the membrane (e.g., *Bacteroides fragilis* enterotoxin, *C. perfringens* α-toxin). Even those toxins that elaborate their effects from within host cells must, at some point, interact with, and pass through, the lipid bilayer of the plasma membrane (e.g., diphtheria toxin, cholera toxin). Finally, both extracellular- and intracellular-acting toxins can modulate eukaryotic signal transduction by interacting with specific receptors on the surface of the plasma membrane (e.g., superantigens, B fragments of cholera toxin).

3.3.2. Eukaryotic Signal Transduction as a Target

A common strategy that pathogenic bacteria employ to modulate specific cellular functions is to produce toxins that directly act upon the factors that are involved in regulating these processes. As mentioned above, toxins sometimes activate signal transduction pathways by functioning as receptor ligands at the plasma membrane. Several other toxins covalently modify "major players" involved in signal transduction, including MAP kinase kinases (e.g., anthrax lethal toxin), heterotrimeric GTP-binding proteins (e.g., cholera toxin, pertussis toxin, and the *Pasteurella multocida* toxin), and members of the Ras superfamily of small GTP-binding proteins (e.g., cytotoxic necrotizing factors and dermonecrotizing toxins). Several toxins act by mimicking eukaryotic molecules involved in signal transduction, including kinases and phosphatases, GTP exchange factors (e.g., *Salmonella* SopE and SopE2 proteins), and the GTPase-activating proteins (*Pseudomonas* ExoS and ExoT, and *Yersinia* YopE). Still other toxins alter cellular signaling by directly catalyzing the overproduction of the important second messenger, cyclic adenosine monophosphate (e.g., the *Bordetella* adenylate cyclase toxins and the anthrax edema toxin).

3.3.3. The Host Cell Cytoskeleton as a Target

The mammalian cytoskeleton is a protein-based mesh underlying the plasma membrane that extends throughout the cytosol. The cytoskeleton is important as a scaffold for numerous intracellular processes including membrane organization and signal transduction. Bacteria produce a number of toxins that modulate cytoskeletal function to promote cellular invasion. Toxins act directly both on the cytoskeleton (e.g., *C. botulinum* C2 toxin, *Salmonella* SipA) and on the small regulatory GTP-binding proteins, Rho, Rac, and CDC42, that control the cytoskeleton (e.g., *Escherichia coli* cytonecrotizing factor, *C. difficile* Toxin A and Toxin B). In addition, toxins act to cause actin polymerization and host cell surface ruffling to facilitate bacterial invasion of mammalian cells.

3.3.4. The Protein Synthesis Machinery as a Target

A number of bacterial toxins target the protein synthesis machinery of eukaryotic cells. There are several different mechanisms by which toxins halt protein synthesis. Diphtheria toxin and *P. aeruginosa* exotoxin A alter the function of the eukaryotic elongation factor-2, which is an essential factor in the elongation step of translation. Other toxins instead modify the ribosomal machinery (e.g., the Shiga and Shiga-like toxins). In general, halting protein synthesis results in the rapid death of intoxicated cells.

3.3.5. Intracellular Membrane Trafficking as a Target

The movement of proteins and other substances throughout the eukaryotic cell requires the trafficking of membrane-bound vesicles. Important cellular processes such as receptor-mediated endocytosis utilize specialized vesicles to internalize portions of the plasma membrane and send them to specific destinations via sorting compartments. Specialized vesicles are also used in exocytosis to transport proteins destined for secretion from the endoplasmic reticulum to the cell surface. Several toxins modulate intracellular trafficking by cleaving important host molecules involved in the fusion of membrane-bound vesicles, with the best-understood example being the Clostridial neurotoxins.

3.3.6. Cell Viability as a Target

In mammalian hosts, both development and homeostasis depend on a tightly regulated balance between cell death and cell growth. Mammalian cells regulate programmed cell death, called apoptosis, by several mechanisms. Not surprisingly, bacteria produce a number of toxins that can induce apoptosis by the activation of cell surface receptors, mimicry of cytosolic secondary messengers, inhibition of protein synthesis, regulation of caspases (the endogenous proteases involved in mediating the apoptotic pathways), disruption of the plasma membrane, and variety of alternative and poorly

understood mechanisms. The induction of apoptosis can benefit a pathogen in a number of ways, including allowing invasive pathogens to escape their intracellular environments, as well as the capacity to control cell turnover rates at mucosal membranes.

3.4. An Overview of Toxin Structure and Function

Clues as to how some toxins interact with their target cells can sometimes be obtained from investigating their structure–function relationships. As we shall see, toxins must be constructed out of the appropriate molecular machinery to successfully intoxicate eukaryotic cells.

3.4.1. Extracellular-acting Pore-forming Toxins

The pore-forming toxins are generally monomeric proteins when exported from bacterial cells. However, these toxins become "functional" when the monomers assemble into complexes of donut-shaped structures that ultimately insert into the membrane, causing the formation of a hole, called a pore or channel, which extends through the entire lipid bilayer. Each toxin monomer interacts "shoulder to shoulder" with at least two other monomers, and contributes structural elements such as beta-barrels (anthrax toxin) or alpha helical assemblies (diphtheria toxin) to form the actual pore scaffold (van der Goot, 2003). Toxin pores and channels are characteristically more hydrophilic within the lumen, and more hydrophobic on the outside facing the fatty acid chains of the lipid bilayer. Insertion of toxin monomers into the lipid bilayer may require significant conformational changes in the toxin structure to expose those elements that are normally not exposed to a more aqueous environment. Depending on the number of monomers that come together to comprise the membrane-spanning complexes, toxin pores and channels range in size from relatively small passages (approximately 10 Å in diameter) to "big gaping holes" (approximately 350 Å in diameter).

3.4.2. Extracellular-acting Degradative Toxins

Members of a second class of extracellular-acting toxins function as hydrolytic enzymes that cleave or degrade molecules either directly on the plasma membrane of host cells or within the extracellular matrix. Prominent examples of degradative toxins include the phospholipases, which destabilize the plasma membrane by catalyzing the cleavage of phospholipids head groups, and the proteases, which cleave proteins at the cell surface and in the extracellular matrix (Titball, 1999).

3.4.3. Intracellular-acting AB Toxins

Intracellular-acting AB toxins are an intriguing group of host cell modulators that comprise two discrete functional domains, which are called the A and B

fragments (Figure 3). In order for AB toxins to elaborate their biochemical activities, they must overcome a significant hurdle, which is transport of the A fragment across the membrane into the cell. Remarkably, the AB toxins are constructed with the molecular machinery to facilitate their own entry into target cells. In general, the B fragment of an AB toxin acts as the delivery or transport vehicle by (1) binding the toxin to the plasma membrane, (2) facilitating toxin entry into the cell, and (3) mediating the translocation of the A fragment into the cytosol. The A fragment is the cargo, which generally functions as an enzyme to catalyze the covalent modification of an intracellular substrate. Neither the A nor the B fragment alone is sufficient to intoxicate

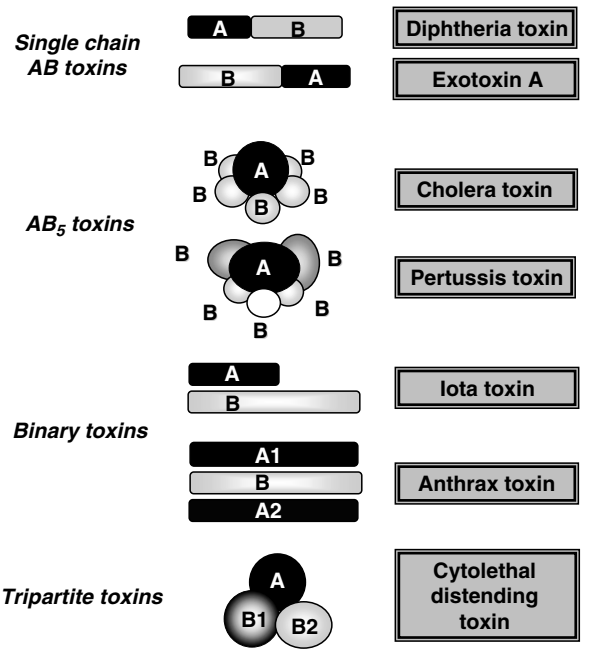

FIGURE 3. Classes of intracellular-acting AB toxins. Intracellular-acting AB toxins have several overall molecular architectures, which represent different organizations and stoichiometries of the A and B fragments. (1) The single chain AB toxins (diphtheria and exotoxin A) are constructed from A and B fragments within a single polypeptide that must ultimately be separated for successful cellular intoxication. (2) The AB_5 toxins are composed of a single A fragment positioned on top of five identical (cholera toxin) or different (pertussis toxin) B fragments arranged in a ring. (3) The binary toxins (iota toxin and anthrax toxin) are composed of discrete A and B fragments that must interact noncovalently for cellular intoxication to be successful. (4) The tripartite toxins (the cytolethal distending toxins) are composed of a single A fragment and two distinct protein subunits that, when assembled, comprise the toxin's B fragment.

host cells. However, the isolated A and B fragments generally retain their biochemical properties as intracellular modulatory factors and delivery vehicles, respectively. There are several characteristic architectures for the AB toxins:

3.4.3.1. Single-chain AB Toxins

The simplest overall AB toxin structure is a single polypeptide comprising both the A and B fragments (Figure 3). Diphtheria toxin and *P. aeruginosa* exotoxin A (ExoA) are examples of single-chain AB toxins. Despite catalyzing the same reaction, the overall domain structures for diphtheria toxin and ExoA are inverted, with the catalytic A fragments localized at the amino terminus of diphtheria toxin but the carboxyl terminus of ExoA. The diphtheria toxin catalytic A fragment is released from the B fragment by cleavage at a protease site and reduction of a disulfide bridge at the interface between the two fragments (Patel et al., 2001).

3.4.3.2. AB_5 Toxins

Other intracellular-acting toxins possess more complicated molecular architectures than the single-chain AB toxins. Members of one prominent class are called the AB_5 toxins, describing toxin complexes comprising a single A fragment associated with five B fragments. The cholera toxin A fragment is centered on top of a donut-shaped complex consisting of five identical B fragments (Figure 3) (Rappuoli and Pizza, 2000). In contrast, *Bordetella pertussis*, the causative agent of whooping cough, secretes an AB_5 toxin comprising a single A fragment and four different B fragments (one is used twice), encoded by five separate genes (Figure 3) (Rappuoli and Pizza, 2000). The A and B fragments are assembled within the periplasmic space prior to the transport of the AB_5 from the bacterium. Similar to diphtheria toxin, the cholera toxin A fragment must be separated from the B complex by a mechanism of activation that will be described later.

3.4.3.3. Binary AB Toxins

The binary AB toxins are also characterized by A and B fragments that are encoded by different genes. However, in contrast to the AB_5 toxins, the A and B fragments of binary toxins are exported as separate polypeptides, and assemble into functional toxin complexes only after associating with the plasma membrane of host target cells (Figure 3). Both the iota toxin of *C. perfringens* and the anthrax toxin from *B. anthracis* are binary AB toxins. Interestingly, the anthrax toxin B fragment, called protective antigen, interacts in binary combinations with two separate A fragments called lethal and edema factors to form lethal and edema toxins, respectively (Rappuoli and Pizza, 2000).

3.4.3.4. Tripartite AB Toxins

In the last 15 years, the discovery of the cytolethal distending toxins (CDTs) produced by several different pathogens has expanded the repertoire of

known AB toxin architectures (Lara-Tejero and Galan, 2002). The CDTs are constructed from the noncovalent association of three peptides, CdtA, CdtB, and CdtC, whose genes are encoded within a single operon (Figure 3). The three-dimensional structure for CDT was recently solved, revealing that the three peptides form noncovalent, but intimate, interactions to form the functional toxin. There are clues that CdtC and CdtA together may comprise the cellular binding moiety, while CdtB may comprise the catalytic A fragment. But the CDT story may not always be so cut and dried, as *Salmonella typhi* was recently demonstrated to generate a molecule that closely resembles CdtB while completely lacking the CdtA and CdtC fragments, which mediate delivery into the cell (Haghjoo and Galan, 2004). How, then, does *S. typhi* CdtB interact with, and enter, host cells? In fact *S. typhi* is hypothesized to deliver the functional CdtB subunit by invasion of the pathogen into the host cell—thus relieving the organism of the need for the CdtA and CdtC subunits (Nesic et al., 2004). This example serves as a reminder that pathogens can solve complex problems—in this case, the delivery of soluble protein toxins across the membrane barrier into cells—in several different ways.

3.4.4. Effectors of Type III and Type IV Secretion Systems

Another class of toxins that elaborate their activities from the inside of target host cells is the toxin effectors of bacterial type III and some type IV secretion systems (T3SS and T4SS). These toxin effectors are functionally equivalent to the enzymatic A fragments of the AB toxins (Figure 3). However, instead of facilitating their own entry into eukaryotic cells, these toxins are directly injected by pathogenic bacteria into target cells using adapted flagellar-based (type III) and conjugative pilin-based (type IV) "molecular syringes." Thus, these injected toxin effectors have no need for the B fragments of AB toxins that are required for delivery into host cells.

3.4.5. Activation of Bacterial Toxins

Toxins are sometimes synthesized as inactive "pro-toxins," which require activation to become capable of intoxicating host cells as fully "mature toxins." The mammalian protease, furin, has been linked to the activation of a number of toxins, including diphtheria and anthrax toxins (Gordon and Leppla, 1994). In the case of diphtheria toxin, the active site of the catalytic A fragment may be partially blocked by a portion of the B fragment. Activation results in the active site of the enzymatic domain becoming unblocked by displacement of the B fragment after furin cleavage at the interface between the A and B fragments, so that the catalytic reaction can occur (Figure 4A). Some toxins must be further activated by the reduction of disulfide bonds that hold toxin fragments together after proteolysis. Diphtheria and cholera toxins are examples of toxins that require the participation of eukaryotic disulfide isomerases to catalyze the reduction of disulfide linkages (Figure 4A)

(Orlandi, 1997). Anthrax toxin is activated by a slightly different mechanism. Recall that anthrax toxin must bind to the plasma membrane surface as the first step in cellular intoxication. However, the toxin's B fragment, called protective antigen, cannot interact with either of the toxin's A fragments until the protective antigen's amino terminus is cleaved off to expose a binding site (Figure 4B) (Lacy and Collier, 2002). Toxins that are activated by mechanisms involving eukaryotic factors are limited, in part, to intoxicating only those cells.

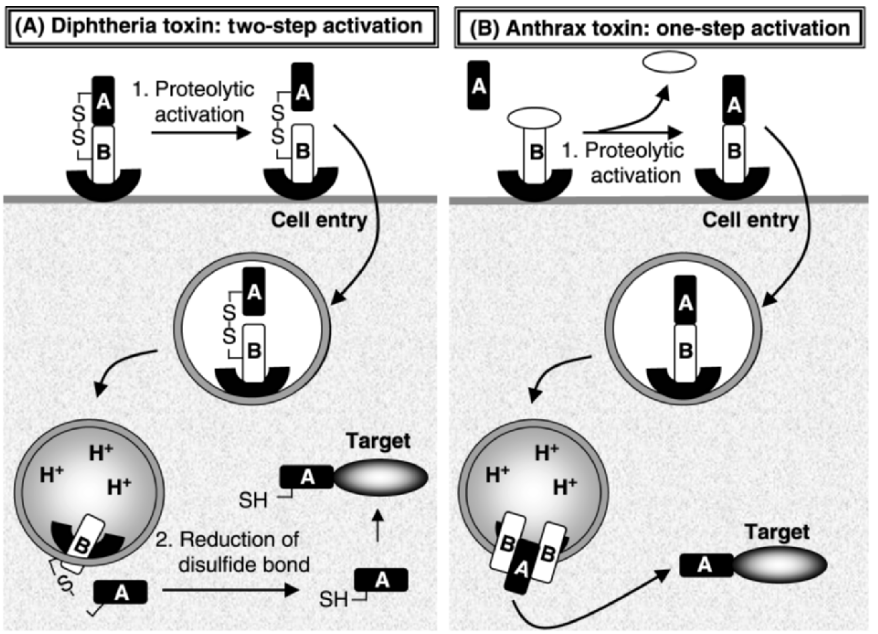

FIGURE 4. Models for toxin activation. (A) The B fragment of diphtheria toxin binds the polypeptide to the cell surface receptor. The single polypeptide is cleaved by the cell surface protease furin, resulting in separate A and B fragments that remain covalently attached by a disulfide linkage spanning the two domains. The toxin receptor complex is internalized by receptor-mediated endocytosis, and the increased acidification of the endosomal compartment results in membrane insertion of the B fragment and translocation of the A fragment across the endosomal membrane into the cytosol. Prior to release of the A fragment from the endosome, the disulfide linkage spanning the A and B fragments is reduced by disulfide isomerase. (B) The A and B fragments of anthrax toxin are synthesized as separate polypeptides. Upon binding to the appropriate cell surface receptor, the amino-terminus of the B fragment is cleaved off by the cell surface protease furin, which exposes a binding site for either of the two toxin A fragments. The AB complex is then internalized into the cell, where the A fragment if subsequently translocated across the endosomal membrane into the cytosol, where it can then modify its eukaryotic target.

4. Genomic Considerations

Many pathogenic bacteria generate toxins to facilitate their lifestyle within the host. But where do pathogens acquire the genes encoding these important factors? In this section, we will briefly discuss the nature and structure of toxin genes.

4.1. The Organization and Nature of Toxin Genes

The structure and organization of toxin genes are diverse. While some toxins are encoded as unlinked genes, or perhaps with regulatory elements, other toxin genes are arranged as part of a larger operon. For example, the genes encoding AB_5 intracellular-acting toxins, such as cholera toxin, are linked within an operon. In contrast, the anthrax toxin genes are not arranged in an

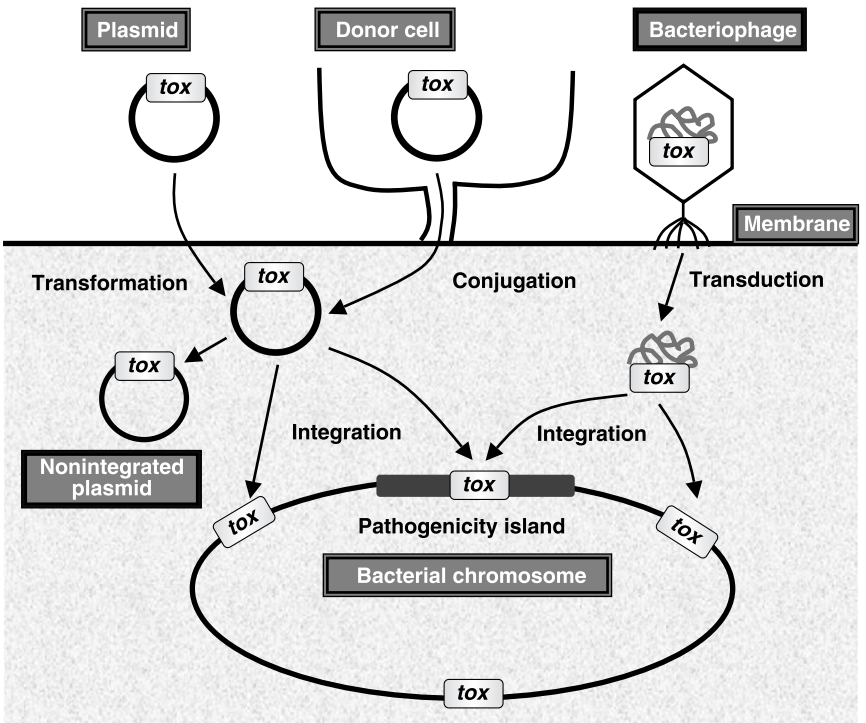

FIGURE 5. The fluid nature of bacterial toxin genes. Toxin genes can be transferred between bacteria of the same and different species on mobile genetic elements, by mechanisms of transformation, conjugation, or transduction. The genes can be integrated into the chromosome, or, if transferred on a plasmid, remain extrachromosomal. Toxins may be unlinked to other genes, or may be part of a larger operon that may or may not be part of a pathogenicity island.

operon, despite the fact that the A and B fragments of this binary toxin must interact for cellular intoxication to occur. While toxin genes are sometimes located within the chromosome, they can also be associated with mobile genetic elements (Figure 5), suggesting that some toxin genes may be transferred between bacteria of the same or different species. Support for the notion of the horizontal transfer of toxin genes comes from the fact that the same, or nearly identical, toxins can be produced by pathogenic bacteria associated with different diseases. Horizontal transfer of toxin genes presumably results in the emergence of new species variants that are better adapted to survive and prosper within specific host niches, which is consistent with the view of toxins functioning as host cell modulators (see Chapter 4).

The acquisition of plasmids harboring toxin genes is an important factor in determining the virulence potential of pathogenic microbes (Figure 5). For example, intestinal *E. coli*, which cause multiple types of diarrheal diseases, are differentiated by pathotype-specific plasmids encoding protein toxins (Clarke, 2001; Dobrindt and Hacker, 1999). Often, virulence plasmids will harbor additional genes necessary for facilitating interactions of toxins with host cells. One illustration of this is found for *Yersinia* spp. that harbor a 70 kb virulence plasmid that includes the virulon encoding the effector toxins as well as the dedicated secretion apparatus for direct injection of the toxin molecules into the host cells. There are many examples of pathogenic bacteria harboring virulence plasmids containing toxin genes, and some of these are listed in Table 2. Interestingly, in the case of *B. anthracis*, the causative agent of anthrax, there is evidence of regulatory cross talk between the chromosome and virulence plasmid harboring the anthrax toxin gene. Such an observation suggests that the virulence plasmid of *B. anthracis* is relatively stable, and this appears to be the case, as little evidence exists to suggest that the virulence plasmid is transferred to even closely related species such as *B. cereus* and *B. subtilis*.

Genes encoding protein toxins sometimes derive from bacteriophages, which are common mobile genetic elements in bacteria (Figure 5). Upon

TABLE 2. Toxin genes encoded on mobile genetic elements.

Mobile element	Toxin gene	Organism
Plasmid	Cytotoxic necrotizing factor-2, cytolethal distending toxin	Enterotoxigenic *Escherichia coli*
	Heat-labile enterotoxin	Enterotoxigenic *E. coli*
	Heat-stable enterotoxin	Enterotoxigenic *E. coli*, enteroaggregative *E. coli*
	Ipa effector proteins	*Shigella* species
	Lethal toxin, edema toxin	*Bacillus anthracis*
	Tetanus neurotoxin	*Clostridium tetani*
	Yop effector proteins	*Yersinia* species
Bacteriophage	Cholera toxin	*Vibrio cholerae*
	Diphtheria toxin	*Corynebacterium diphtheriae*

entering a bacterium, bacteriophages carrying toxin genes will sometimes integrate into the chromosome. The diphtheria toxin gene was first discovered to be derived from a bacteriophage (corynephage β) when pathogenic strains of *Corynebacterium diphtheriae* were determined to be lysogenic, while avirulent strains are nonlysogenic (Table 2) (Barksdale and Pappenheimer, 1995). Interestingly, while the ability of *C. diphtheriae* to cause diphtheria toxin-mediated disease is mainly determined by the presence of the lysogenic prophage, the factor primarily responsible for regulating toxin production is unlinked and on the chromosome, suggesting that there is extensive "cross talk" between the chromosome and the corynephage β (Boyd et al., 1990).

Mobile genetic elements can serve as precursors for the evolution of stable pathogenicity islands (Figure 5). Toxin genes localized to pathogenicity islands have been described for both Gram-negative and Gram-positive bacteria. For example, pertussis toxin is encoded by genes from the *ptx-ptl* locus of *B. pertussis* (Antoine et al., 2000). In addition, the metalloprotease toxin called fragilysin from *B. fragilis* is derived from the fragilysin pathogenicity islet (Moncrief et al., 1998). A third example is the *Staphylococcus aureus* toxic shock syndrome toxin 1 (TSST-1), which is encoded by a gene located on two pathogenicity islands, SapI1 and SapI2 (Lindsay et al., 1998). Toxins that are injected directly into target cells are often encoded by genes that may be linked to other genes encoding the actual injection machinery (e.g., T3SS and some T4SS). For *Salmonella*, pathogenic *E. coli* strains, the gastrointestinal pathogen *H. pylori*, as well as other pathogens, genes encoding toxin effectors linked to T3SS or T4SS are typically clustered within pathogenicity islands (Backert et al., 2003; Waterman and Holden, 2003).

4.2. Where Do Bacterial Toxins Come From?

As just discussed, toxin genes can clearly move between bacteria to allow them to rapidly take on new virulence properties. But where did toxin genes originally come from? How did toxins evolve? There are several lines of thought about these questions, although the "correct" answers, by the very nature of the inquiries, are difficult to come by.

Recall that toxins often act upon specific eukaryotic molecules, which can result in profound modulatory effects within intoxicated cells. For example, diphtheria toxin kills mammalian cells by selectively modifying a unique posttranslationally modified amino acid (diphthamide) of eukaryotic elongation factor-2 (EF-2), resulting in the cessation of protein synthesis and subsequent cell death (Figure 6). An obvious question is, therefore, how did the diphtheria toxin catalytic fragment derive selectivity for diphthamide, which is never found in prokaryotic cells? The origin of target specificity is a puzzling aspect underlying the action of many toxins, especially those that act from inside eukaryotic cells. What selective pressure drives the evolution of enzyme specificity for eukaryotic substrates? In addition to targeting molecules that are uniquely eukaryotic, some bacterial toxins exert enzyme

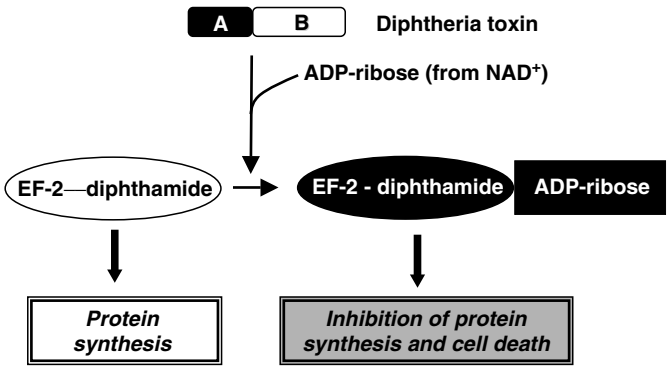

FIGURE 6. Diphtheria toxin mode of action. The A fragment of diphtheria toxin catalyzes the ADP-ribosylation of a unique, posttranslationally modified histidine residue on eukaryotic elongation factor-2 (EF-2). This single covalent modification alters the function of EF-2, resulting in the cessation of protein synthesis and subsequent cell death.

activities that were previously believed to be found exclusively in eukaryotic cells. Collectively, such observations suggest the intriguing hypothesis that some toxins may be of eukaryotic origin.

Conversely, it is possible that some toxins, although bacterial in origin, descended from proteins targeting other bacteria. Competition between different bacterial species for scarce nutrients can be fierce. It is thus tempting to speculate that toxins that act by punching holes in membranes of other cells, or by entering and killing other cells, may have descended from the bacterial colicins, a class of bacterial "toxins" that target and kill other bacterial species.

5. Timing and Location Is Everything: Bacteria Regulate When and Where Toxins Are Produced

Pathogens have exquisite systems for regulating the production of toxins. Bacteria sense dynamic changes that occur as they pass through the host, and express both chromosomal- and extrachromosomal-derived toxin genes in those times and places where toxin production is most beneficial to the bacterium. The regulation of toxin production is important to the bacterium because the energy needed to both synthesize toxins and export them across bacterial membranes is costly. While the mechanisms of virulence factors regulation are discussed in Chapters 12 and 13, it is of interest to briefly mention several of the overall strategies used by pathogens to regulate toxin production.

5.1. *Two-Component Regulatory Systems of Toxin Production*

Toxin expression is often controlled by bacterial two-component regulatory systems, which are widely conserved in both Gram-positive and Gram-negative species. Two-component systems comprise transmembrane sensors and cytosolic response regulators. The activation of a single sensor molecule by an environmental signal can result in signal amplification through the action of multiple response regulators, which interact with the *cis* regulatory regions upstream of the toxin gene to activate transcription of toxin genes (Figure 7).

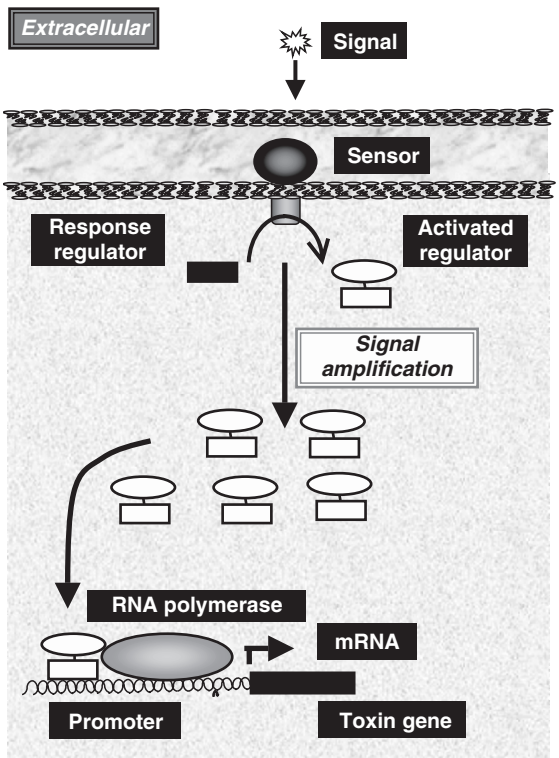

FIGURE 7. Two-component regulation of toxin genes. Many bacteria sense external signals via a sensor, which is an integral membrane protein (shown here as the inner membrane of a Gram-negative bacterium). Conformational changes occur within the transmembrane domain of the sensor, causing a cytosolic domain to phosphorylate a second protein within the cytosol called the response regulator. The initial external signal is amplified because the sensor can activate multiple response regulator molecules. The activated response regulator molecules can interact with *cis*-elements of the regulatory regions of specific genes to regulate the appropriate gene expression that occurs in response to the initial external signals.

Examples of bacterial toxins controlled at the transcriptional level by two-component regulatory systems include those made by *C. perfringens*, *S. aureus*, and *Streptococcus pyogenes*. One of the best-studied examples of two-component regulation of bacterial toxins is found in *B. pertussis*. In response to the appropriate environmental signals, the sensor BvgS activates the response regulator BvgA to control the expression of three different toxins: pertussis toxin, adenylate cyclase toxin, and dermonecrotizing toxin (Konig et al., 2002). Interestingly, this sophisticated system turns on toxin genes while simultaneously turning off other genes that are detrimental to *B. pertussis* when colonizing the host.

5.2. Regulation of Toxin Production by Environmental Iron

Toxin production is sometimes regulated by the levels of accessible iron within the host (Litwin and Calderwood, 1993). Sequestering of iron by the host is an important strategy to attenuate the growth of pathogenic bacteria, and, in response, the bacteria use multiple counterstrategies for acquiring iron in order to survive. Low iron concentrations can act to trigger the expression of toxins that kill host cells, thereby causing the release of intracellular iron for use by the invading microbes.

One of the best-understood examples of iron-based toxin regulation is the control of diphtheria toxin production by *C. diphtheriae* (Figure 8). DtxR is an iron-responsive repressor that, in the presence of iron, binds to the operator region of *tox* (the structural gene for diphtheria toxin) to prevent transcription (Tao et al., 1994) (Figure 8). However, under limiting iron conditions, DtxR dissociates from the operator, allowing transcription of *tox*. Other examples of iron-regulated toxins include Shiga toxin and *Pseudomonas* exotoxin A, which have similar pattern of expression, but with more complex control mechanisms.

5.3. Regulatory Systems Shared by Virulence and Nonvirulence Genes

Toxin expression in several pathogens is controlled by mechanisms that are common to regulating both virulence and nonvirulence genes. In particular, toxin production may occur, along with numerous other bacterial genes, in response to stress. The AraC proteins are a diverse family of proteins that control expression of bacterial genes involved in an array of functions, including stress-response. Recently, AraC family members have been discovered to also contribute to the regulation of some toxins, especially those effectors that are directly injected by some pathogenic bacteria into host cells by T3SSs (Francis et al., 2002). A specific example is the ExsA protein of *P. aeruginosa*, an AraC-like protein, which, in coordination with a number of auxiliary factors, positively regulates transcription of four toxins, as well as

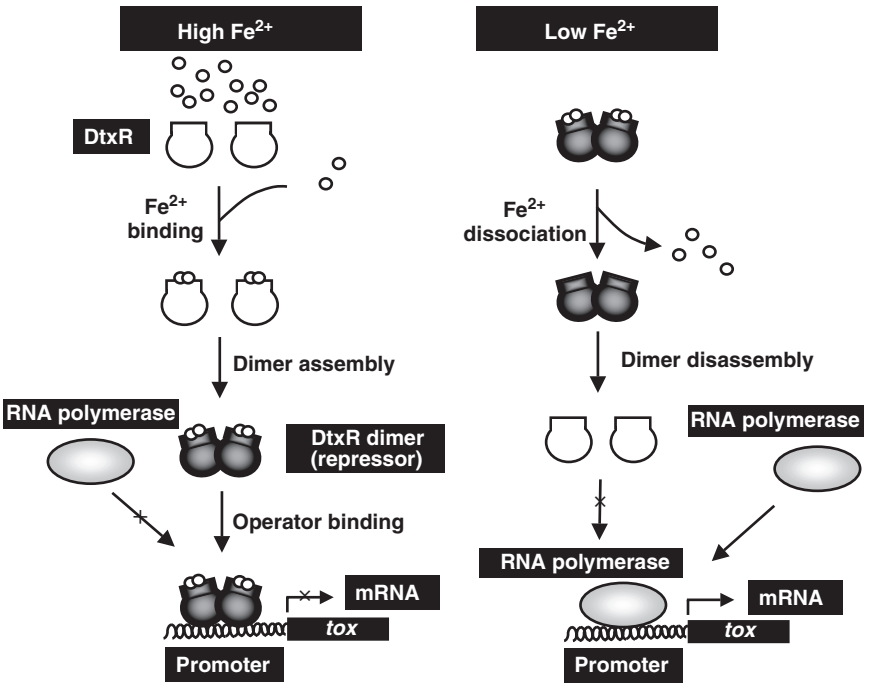

FIGURE 8. Iron regulation of diphtheria toxin production. Under conditions of high iron, DtxR binds two atoms of ferrous iron, and forms a dimer that has high affinity for the *tox* operator to repress transcription of *tox*. Under conditions of low iron, ferrous iron dissociates from DtxR, causing a conformational change such that DtxR now has relatively low affinity for the *tox* operator. Under these conditions, RNA polymerase readily binds to the promoter, and transcription of *tox* occurs.

the type III injection apparatus needed to translocate these toxins into target cells. While the detailed mechanisms by which AraC family members regulate toxin production is not entirely understood, the involvement of such proteins suggests that some toxins may be produced in response to stress-related signals, which is consistent with the idea of pathogenic bacteria encountering stress within the environment of the host.

5.4. Quorum Sensing

The relationship between quorum sensing and toxin production has been established in several pathogenic bacteria (see Chapter 11). For example, during infection with *P. aeruginosa*, the important virulence factor exotoxin A is not produced until the bacteria reach sufficient density to affect the host and benefit the entire bacterial population (Gambello et al., 1993; Passador et al., 1993). Inappropriate "early" expression of a virulence factor such as exotoxin A may be detrimental to the pathogen by alerting the host to deliver a

response that a small bacterial population may not be able to withstand. In contrast to *P. aeruginosa*, quorum sensing in *V. cholerae* represses, rather than activates, the expression of cholera toxin (Camara et al., 2002). Although the relationship of *V. cholerae* quorum sensing systems to virulence is not entirely understood, the downregulation of virulence genes (including the cholera toxin gene) by quorum sensing at high cell density has been proposed to promote the detachment of bacteria from intestinal epithelial cells to facilitate the spread of the pathogen from person to person (Camara et al., 2002).

5.5. Posttranslational Regulation of Toxin Activity

While toxins are regulated primarily by the transcriptional control of gene expression, several toxins are further regulated by posttranslational mechanisms. The catalytic functions of two intracellular-acting toxins have been demonstrated in vitro to be "activated" by direct interactions of the toxins with two specific mammalian factors. The cholera toxin catalytic A fragment, which covalently modifies the heterotrimeric G protein, Gs, demonstrates enhanced catalytic activity in the presence of adenosine diphosphate-ribosylation factors (ARFs) (Randazzo et al., 2000). Interestingly, ARFs are one branch of the Ras superfamily of guanosine triphosphate (GTP)-binding proteins, which regulate membrane traffic and the actin cytoskeleton. ARFs function both constitutively within the secretory pathway and as targets of signal transduction in the cell periphery. A second example is the intracellular effector toxin ExoS, from *P. aeruginosa*, which modifies Ras (Barbieri, 2000). The ADP-ribosylation activity of this toxin is dependent on the cofactor 14-3-3 (Coburn et al., 1991). The family of 14-3-3 proteins play an important role in signal transduction pathways by interacting with other signal proteins, such as Raf and KSR (kinase suppressor of Ras).

Another example of posttranslational regulation of toxin activity is the selective modification of toxins. Adenylate cyclase toxin from *B. pertussis* is an intracellular-acting toxin that requires posttranslational acylation of a specific lysine residue in order to transport its catalytic A fragment to the target cell interior (Ladant and Ullmann, 1999). In addition, modification with the lipid moiety is necessary for the toxin to form transmembrane channels required for hemolytic activity.

6. Delivering the Goods: Exporting Toxins Out of Bacterial Cells

Following synthesis, toxins must be exported out of bacterial cells to gain access to their host targets. This is a nontrivial issue, as newly synthesized toxin polypeptides must be able to fold into their native conformations, sometimes assemble into higher-ordered complexes in a manner that does not impede movement out of cells, and traverse multiple barriers of the bacterial

outer envelope. In Gram-negative bacteria, these barriers are the cytoplasmic membrane, a thin layer of peptidoglycan, and the lipopolysaccharide-rich outer membrane. The entire process of toxin export requires the involvement of specific accessory proteins to properly direct the toxins out of the cell, and is costly to the cell in terms of energy.

From the standpoint of host–pathogen interactions, two potential outcomes of toxin export from bacterial cells are important to consider (Figure 9). Toxins are exported either into the extracellular environment or, alternatively, directly into target cells (Figure 9). These two outcomes have important implications as to how toxins interact with, and modulate, host cells.

Toxin export is perhaps better understood in Gram-negative than Gram-positive bacteria. There has been agreement in classifying the overall Gram-negative export mechanisms into five different secretion pathways referred to as type I–type V secretion, respectively (Figure 10). These export systems are discussed in detail in Chapter 9, and we will only briefly discuss the different mechanisms to emphasize the importance and consequences of toxin export. Mechanistic overlaps in the five overall secretion pathways exist

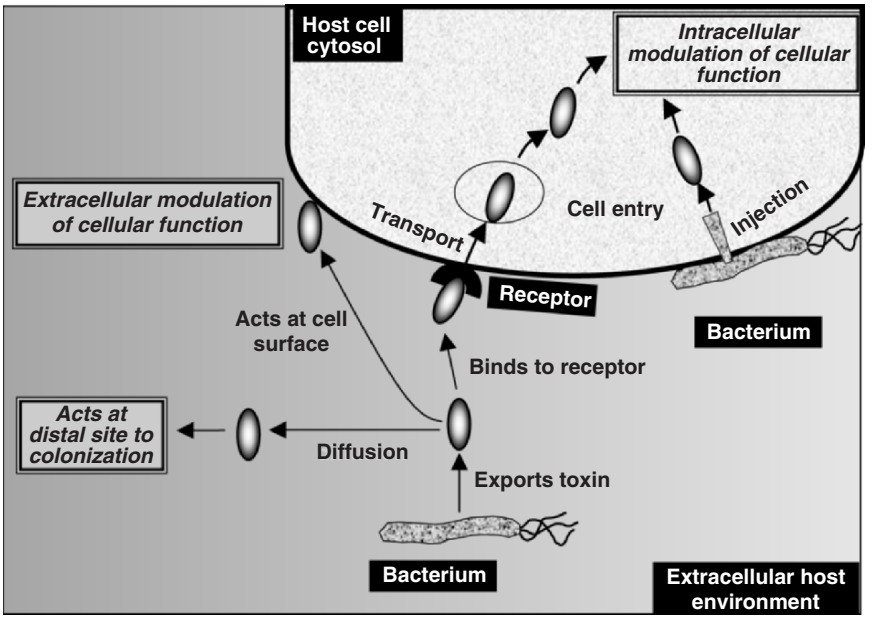

FIGURE 9. Outcomes of toxin export. Toxin export via a type III or a type IV system results in the direct injection of toxin effectors into the cytosol, so that only the eukaryotic cell that a bacterium is in direct contact with will be modulated. Toxin export into the extracellular environment of the host results in modulation of those cells at the site of infection as well as those distant from the site of infection. These toxins act from either the outside or the interior of eukaryotic cells.

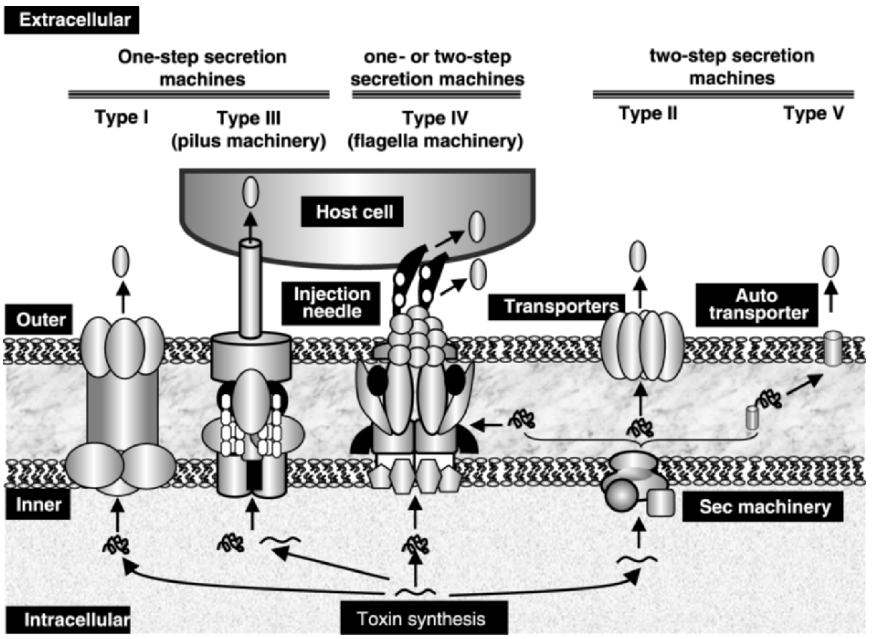

FIGURE 10. Toxin export in Gram-negative bacteria. Following synthesis, toxins can be exported directly into the extracellular environment (types I, II, IV, and V secretion mechanisms) or directly into the eukaryotic cell (types III and IV secretion mechanisms). Toxins can be exported directly across the inner and outer membranes (types I, III, and IV secretion mechanisms), or may be exported in two steps involving time spent in the periplasmic space (types II, IV, and V secretion mechanisms).

and, in addition, one pathway (type IV) can apparently export different toxins either into the extracellular host environment or directly into host cells.

6.1. Export of Toxins into the Extracellular Host Environment

T1SS, T2SS, T5SS, and in at least one case, T4SSs export toxins from the bacterial cell into the extracellular host environment. Export into the extracellular milieu of the host might seem, at first glance, to be rather inefficient for modulating cells in the immediate vicinity of colonization because secreted toxins can either diffuse away or be washed from the site of bacterial export. Moreover, the "effective concentrations" of toxins are likely to decrease dramatically the farther the toxin has strayed from the site of bacterial export, which is important when considering the capacity of toxins to successfully intoxicate host cells.

Despite these apparent drawbacks, the export of some toxins into the extracellular environment within the host may, in fact, be required to elicit

the maximal benefits for a pathogen. Intoxication of sensitive cells far away from the site of bacterial colonization within the host may result in toxins having the capacity to exert a wider sphere of influence on cells and tissues. For example, toxins can facilitate the persistence of pathogens by modulating immune cells located far from the site of infection. Such a role has recently been suggested for the vacuolating cytotoxin (VacA) of *H. pylori*, which is the causative agent of gastric ulcer disease in humans (Gebert et al., 2003). *H. pylori* infections persist for years at the gastric mucosa, in part, because of the capacity of the bacterium to alter both the innate and adaptive immune responses, although the bacteria may never directly contact lymphocytes located beneath the epithelial barrier in the stomach. However, VacA was recently shown to suppress the proliferation of activated T-helper cells, thereby attenuating the appropriate immune response (Gebert et al., 2003). The implication of this finding is that during *H. pylori* infection, VacA must traverse a considerable distance across the epithelial membrane to intoxicate the T lymphocytes.

Let us now look briefly at the overall mechanisms used by Gram-negative bacteria to export toxins into the extracellular environment of the host.

6.1.1. Type I Secretion of Toxins

The simplest export mechanism is the direct transport of toxins across the inner and outer membranes into the extracellular environment. Such a "one-step" export mechanism is known as type I secretion or, alternatively, the ATP-binding cassette (ABC) transporter pathway. The type I cellular secretion apparatus is relatively simple and consists of three components that assemble into a translocation tunnel for direct passage of toxins through the bacterial outer envelope (Figure 10). Toxins transported by the type I apparatus cannot undergo the additional "processing" that generally takes place within the periplasmic space. Pathogenic *E. coli* hemolysin A (HlyA), which acts on the membrane of mammalian cells, is one of best-characterized examples of a toxin that is exported by type I secretion (Koronakis et al., 2003; Lee and Schneewind, 2001).

6.1.2. Type II Secretion of Toxins

Upon clearing the hurdle of the Gram-negative cytoplasmic membrane, some toxins need to make a "pit-stop" in the periplasm prior to transport across the outer membrane (Figure 10). The periplasm is a special compartment that is highly conducive to protein folding and modification, as well as the assembly of complex toxins (e.g., AB_5 toxins). Type II, type V, and at least one variation of type IV secretion are examples of "two-step" toxin export mechanisms. In each case, toxin secretion across the inner membrane requires the sec apparatus, which consists of a cytoplasmic secretion specific–chaperone, a protein translocation ATPase, and an integral membrane protein complex with at least six protein subunits. After synthesis in the cytoplasm, toxins

are targeted for sec-dependent secretion via their amino-terminal signal peptides prior to folding into their functional structures. After transport across the inner membrane, the signal peptides are cleaved off, and the mature polypeptides are released into the periplasm. At this point, the mechanisms for crossing the outer membrane diverge to comprise type II, type V, and at least one case of type IV secretion.

Type II secretion is often referred to as the main terminal branch of the general secretion pathway (Stathopoulos et al., 2000). Notably, type II cellular machinery for passage through the periplasm and outer membrane is substrate-specific. Examples of bacterial toxins that are exported via the type II secretion pathway include cholera toxin, *P. aeruginosa* exotoxin A, and *Aeromonas hydrophila* aerolysin.

6.1.3. Type IV Secretion of Toxins in the Extracellular Host Environment

Toxins that are exported by type IV pathways use conjugative pilin-like structures as secretion devices (Burns, 2003) (Figure 10). At least one toxin, pertussis toxin from *B. pertussis*, uses type IV secretion as a means to be exported from the bacterium into the extracellular host environment (Burns, 2003). Although the mechanism remains poorly understood, one model suggests that the separate polypeptides of pertussis toxin, which comprise an AB_5 toxin, are secreted initially into the periplasm. The pilus structure has been proposed to function as a "piston" to push the assembled toxin within the periplasm across the outer membrane.

6.1.4. Type V Secretion of Toxins

Some toxins do not rely on cellular machinery to transverse the Gram-negative outer membrane, but instead are able to transport themselves across this barrier by a mechanism referred to as type V secretion (Figure 10) (Stathopoulos et al., 2000). Type V secretion substrates, which are referred to as autotransporter proteins, are first transported across the inner membrane by a sec-dependent mechanism. Once the amino-terminal signal peptide of the precursor is cleaved within the periplasm, the carboxyl-terminal domain of the protein inserts into the outer membrane and forms a pore-like structure, which mediates the transit of the amino-terminal portion (the passenger domain) from the periplasm to the cell surface. The carboxyl-terminal "transporter" domain of the protein is cleaved, and the mature form of the toxin is released into the extracellular environment (Stathopoulos et al., 2000). Presumably, toxins exported by the type V mechanisms have a potential advantage of not requiring substrate-specific cellular components for successful transport. Examples of type V autotransporters include the IgA1 proteases of *Neisseria* and *Haemophilus*, which were the first autotransporters identified (Henderson et al., 1998). Additional examples include the hemolysins of *Proteus mirabilis* and *Serratia marcescens*, the *H. pylori* vacuolating cytotoxin (VacA), and the *Shigella flexneri* IcsA protein. Finally,

members of the *Enterobacteriaceae* generate autotransporters with serine protease activities (known as the SPATEs), including the temperature-sensitive hemagglutinin from pathogenic *E. coli*, the EspP protein of Shiga toxin–producing *E. coli*, as well as SepA, SipA, and Pic from *Shigella* spp. (Henderson and Nataro, 2001; Lee and Schneewind, 2001).

6.2. Direct Export of Toxins into the Host Cell Cytosol

Intracellular-acting toxins elaborate their modulatory effects from within the cytosol of target cells (Figure 10). For the AB intracellular-acting toxins, export into the extracellular host environment may allow pathogens to modulate cells and tissues that are located far away from the actual site where the toxin was exported. However, bacteria sometimes need to modulate only those cells with which intimate contact has been established. To selectively modulate target cells, some pathogens directly inject toxins into the cytosol of eukaryotic cells using adapted flagella or pili as molecular syringes by what is referred to as type III or type IV secretion, respectively. Protein toxins delivered by these pathways are sometimes called type III or type IV "effectors."

6.2.1. Type III Secretion of Toxins

Several Gram-negative pathogens have adapted flagella-like structures for the export of toxins (Lee and Schneewind, 2001; Plano et al., 2003). The type III apparatus, which is a complex of at least 20 different proteins (Figure 10) (Thomas and Brett Finlay, 2003), resembles a "needle." The assembled "injectosome" promotes the direct transport of toxins through a hollow core from the cytosol of the bacterial cell across the periplasm and outer membrane, similar to that of type I secretion (see Chapter 9). However, whereas toxins secreted by a type I mechanism end up in the extracellular host environment, toxins secreted by a type III secretion mechanism are exported directly into the host cell cytosol. Both the biogenesis and the structure–function relationships of the type III injectosomes are described in Chapter 9.

Type III secretion was first identified in *Yersinia* spp., and now has been recognized in many human, animal, and plant pathogens (Plano et al., 2003). Well-characterized toxin effectors include the Yop proteins from *Yersinia*, Sop proteins from *Salmonella*, and Ipa proteins from *Shigella*. The toxin effectors of T3SSs seem to be well evolved for modulating target proteins, as they sometimes elaborate enzymatic activities more often found in eukaryotic cells than the prokaryotic cells from which they originated.

6.2.2. Type IV Secretion of Toxins

Type IV systems are capable in some cases of transporting toxins directly into eukaryotic cells. The number of confirmed and putative T4SSs associated with pathogenic bacteria, such as *B. pertussis*, *Brucella* spp., *Bartonella henselae*, *Helicobacter* spp., and *Campylobacter* spp., has rapidly increased in recent

years. While the genes encoding the actual secretion apparatus are identifiable by sequence conservation, pin-pointing the exported effector toxins has been more of a challenge. CagA, from the gastric pathogen *H. pylori*, was the first effector demonstrated to be transported into mammalian cells by a one-step, type IV mechanism (Backert et al., 2003; Nagai and Roy, 2003).

6.3. Toxin Secretion from Gram-positive Bacteria

Mechanisms of toxin export from Gram-positive bacteria are relatively understudied compared to those from Gram-negative bacteria, despite the fact that some of the most actively investigated toxins, including anthrax and diphtheria toxins, originate from Gram-positive pathogens. Like their Gram-negative counterparts, Gram-positive bacteria use a sec-dependent pathway for transport of toxins across the cytoplasmic membrane, although significant differences exist. In recent years, more specific toxin secretion systems such as the twin-arginine translocation (Tat) pathway, the polypeptide translocating ABC transporters, and the type III flagella-like and the type IV prepilin-like pathways have been identified in Gram-positive bacteria (Tjalsma et al., 2000). The identification of such secretion systems suggests that Gram-negative and Gram-positive bacteria may share similar strategies for releasing modulatory factors that act upon target cells. For example, a system analogous to type III secretion, allowing direct injection of toxin effectors into target cells was recently identified in *S. pyogenes*, a causative agent of throat, soft tissue, and systematic infections (Gauthier and Finlay, 2001). The bacterium may use a toxin, streptolysin O, which forms large pores, or holes, in the plasma membrane of target cells to translocate effectors into the host cell cytosol. However, the importance, if any, of these toxin-mediated translocons for the export of other toxin effectors out of Gram-positive bacteria remains to be determined.

7. Toxin Interactions with Host Target Cells

Modulation of host properties requires productive interactions between toxin molecules and sensitive eukaryotic cells. For those toxins that act upon molecular targets found only inside host cells, injection into the cytosol of eukaryotic cells by T3SS or T4SS is an efficient and direct means to access the intracellular environment. However, many toxins are secreted into the extracellular environment, where the journeys to their specific targets can be significantly more arduous. To begin with, toxins must sometimes navigate the treacherous extracellular matrix, which is loaded with degradative proteases, to locate sensitive target cells. The sensitivity or resistance of a particular host cell type to a specific toxin often depends on the presence or absence of plasma membrane components that can function as toxin receptors. Some toxins can elaborate their effects directly at the plasma membrane of target

cells, whereas the journey has just begun at the plasma membrane for the intracellular-acting AB toxins, which must enter cells to ultimately modify their molecular targets. The AB toxins face the nontrivial problem of traversing the eukaryotic cell membrane barrier, which is a thermodynamically unfavorable process. While there are a few examples of toxins apparently translocating directly from the plasma membrane into the cytosol, such a mechanism is the exception rather than the rule. Most intracellularly acting toxins are first internalized by the normal endocytic mechanisms used by mammalian cells to import proteins. However, as we shall examine, internalized toxins must still somehow exit the endocytic pathways to access their targets within the cytosol. Let us now look in more depth at the different steps involved in cellular intoxication.

7.1. Host Cell Receptors Mediate Toxin Interactions with Target Cells

To successfully intoxicate host cells, toxins generally must interact with specific plasma membrane receptors. The importance of the toxin/receptor complex to toxin action is poignantly demonstrated by the observation that cells lacking toxin receptors are essentially resistant to those toxins (Naglich et al., 1992). The significance of toxin/receptor binding immediately raises the obvious question of why host cells retain receptors for bacterial toxins. At the very least, bacterial toxins have seemingly "hijacked" existing receptors that must contribute in some manner to normal cellular functions. An equally puzzling question is how toxins, which are generated by prokaryotic microbes, are capable of discriminating for specific eukaryotic receptors. Hints as to possible answers may be gleaned from considering the molecular nature of some toxins. The high-resolution structure of diphtheria toxin provides the striking picture of a protein with three, clearly distinct, functional domains, corresponding to the receptor-binding and translocation domains (which together comprise the toxin's B fragment), and the catalytic domain (the toxin's A fragment) (Choe et al., 1992). Each of these three domains possesses distinct secondary and tertiary structural motifs and, remarkably, can function biochemically as independent entities when the toxin is artificially divided into three discrete proteins (although all three domains are required as intact toxin to successfully intoxicate cells). That is, the A fragment is an active enzyme that catalyzes the modification of its eukaryotic target, the translocation domain interacts with membranes, and the receptor-binding domain binds to the diphtheria toxin receptor. These observations suggest that diphtheria toxin was originally fashioned by first recruiting, and then splicing together, preexisting genes encoding the discrete functional domains into the gene that encodes the fully competent toxin proteins. Because the toxin's receptor-binding domain presumably mimics the eukaryotic ligand that normally engages the cellular receptor, it is tempting to speculate that the gene encoding the diphtheria toxin receptor-binding domain may have been eukaryotic in origin.

7.1.1. Classes of Toxin Receptors

High-affinity toxin receptors are generally plasma membrane proteins, glycoproteins, or glycolipids (Table 3). Diphtheria toxin binds to a transmembranous heparin-binding protein, which is the precursor form of the epidermal growth factor–like growth factor (Saelinger, 2003). The protective antigen of anthrax toxin was recently shown to bind to a so-called type I membrane protein (anthrax toxin receptor, ATR), which is a highly expressed ubiquitous molecule on cell surfaces (Bradley and Young, 2003). The receptor for *Pseudomonas* exotoxin A is a glycoprotein, LRP (low-density lipoprotein receptor-related protein) (Saelinger, 2003). A number of bacterial toxins bind to glycosphingolipids, and in particular to gangliosides, which are acid glycosphingolipids. For example, cholera toxin binds to the ganglioside GM1, tetanus toxin binds to GT1 and/or GD1b, botulinum toxins binds to GQ1b, and delta toxin interacts with GM2. Other toxins including Shiga toxin bind

TABLE 3. Toxin receptors.

Bacterial toxin	Toxin receptor
Aerolysin (*Aeromonas hydrophila*)	Glycosylphosphatidyl inositol (GPI)-anchored proteins
Anthrax toxin (*Bacillus anthracis*)	Tumor endothelial marker 8 (TEM8) capillary morphogenesis protein 2 (CMG2)
Botulinum toxin (*Clostridium botulinum*)	GT1b and GQ1b
Cholera toxin (*Vibrio cholerae*)	GM1 (high affinity) and GD1b (low affinity)
Delta toxin (*Clostridium perfringens*)	GM2
Diphtheria toxin (*Corynebacterium diphtheriae*)	Heparin-binding epidermal growth factor–like growth (HB-EGF) and CD9
Exotoxin A (*Pseudomonas aeruginosa*)	Alpha 2-macroglobulin receptor (CD91)/low-density lipoprotein receptor-related protein (LRP)
Heat-labile toxin (*Escherichia coli*)	GM1 and GD1b (low affinity), galactosides
Heat-stable toxin (*Escherichia coli*)	Guanylyl cyclase C
Shiga toxin/shiga-like toxins (*Shigella flexneri* and *Escherichia coli*)	Glycosphingolipid globotriaosylceramide (Galalpha1–4Galbeta1–4 glucosyl ceramide, Gb3)
• STX 1, STX 2	Globotetraosylceramide (GalNAc beta1–3 Galalpha1–4Galbeta1–4 glucosyl ceramide)
• STX 2e	
Superantigens (several pathogens)	Major histocompatibility complex class II and T cell receptor (Vβ or Vγ)
Tetanus toxin (*Clostridium tetani*)	GT1 and/or GD1b & GQ1b
Adenylate cyclase toxin (*Bordetella* species)	CD11b, a β2 integrin receptor
Vacuolating cytotoxin (*Helicobacter pylori*)	RPTP-alpha, RPTP-beta, heparin/heparan sulfate (H/HS), EGFR
Cytotoxic necrotizing factor 1 (*Escherichia coli*)	Laminin receptor precursor (LRP)
Alpha-hemolysin (HlyA) (*Escherichia coli*)	Glycophorin

to the globoside globotriaosyl ceramide (GB3), which is a neutral glycosphingolipid (Saelinger, 2003). Finally, some toxins may interact with multiple plasma membrane components. For example, the *Clostridial* neurotoxins bind to a ganglioside and a protein that may function as a receptor complex on target cells (Saelinger, 2003).

7.1.2. Consequences of Receptor Binding

The requirement for receptor binding can often dictate how sensitive a particular eukaryotic cell type is to a specific toxin. An important point worth repeating is that cells lacking receptors for a particular toxin may be resistant to the modulatory or lethal action of that toxin. But how does receptor binding contribute to toxin function? To begin with, receptor binding may act to effectively "concentrate" toxin molecules at the plasma membrane, which is particularly significant if in vivo toxin concentrations are very low within the host. Tethering of the toxin to the plasma membrane can also result in several different downstream consequences, including (1) receptor-mediated endocytosis into the cell; (2) the activation of cellular signaling pathways; (3) the cross-linking of certain cell types; and (4) the facilitation of membrane insertion.

7.1.2.1. Receptor Binding Can Promote Uptake of Toxins that Act Intracellularly

The first step in the arduous journey of bacterial AB toxins to their intracellular molecular targets is binding to receptors on the plasma membrane of host cells (Figure 11). Toxin receptors serve to tether the B fragment to the plasma membrane, often with high affinity, so that both the A and B fragments can be internalized within membrane-bound vesicles by the process of receptor-mediated endocytosis, which is normally used by mammalian cells to internalize proteins. The uptake and trafficking of toxins to the appropriate cytosolic "entry portals" are important steps for the cellular modulatory activities of most AB toxins.

7.1.2.2. Receptor Binding Can Modulate Host Signal Transduction

Mammalian cells sense their immediate environment by signal transduction processes, which are triggered by the binding of exogenous ligands, such as hormones, to plasma membrane receptors. Not surprisingly, some pathogens modulate host cells by exporting toxins that function as receptor-binding ligands to induce mammalian signal transduction. Enterotoxins secreted by several enteric bacterial species interact with the intestinal mucosa and modulate eukaryotic signal transduction by binding to specific cell surface receptors. The best-studied examples are the *E. coli* heat-stable (ST) enterotoxins, which can stimulate intestinal secretion resulting in diarrhea. The STs bind to epithelial cell receptors belonging to a family of receptor cyclases. The interaction of ST and guanylyl cyclase C leads to signaling across the cell membrane into the cell, while the toxin remains at the cell surface. The result of

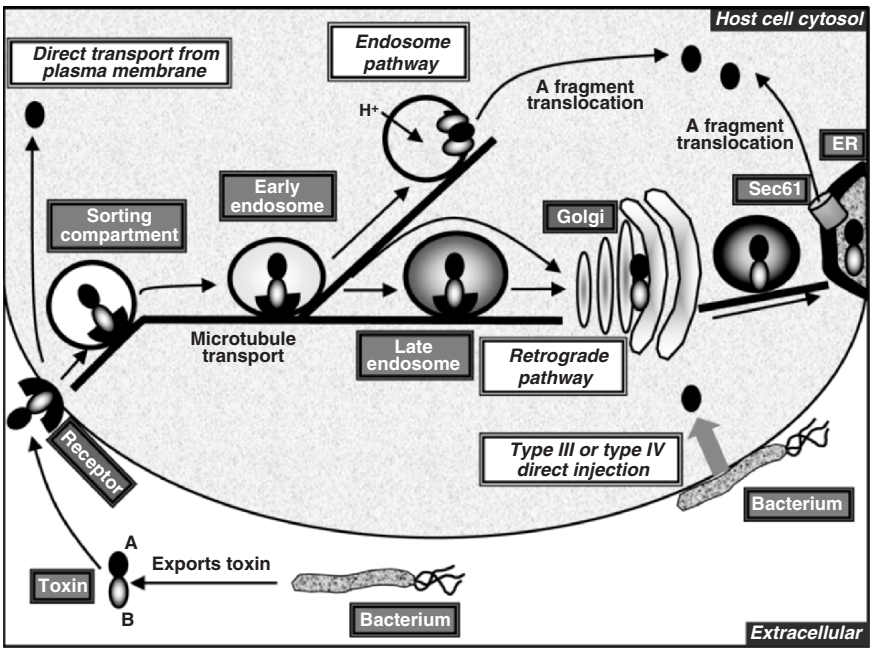

FIGURE 11. Strategies of toxin entry, trafficking, and translocation. Some bacteria export toxins directly into the cytosol of eukaryotic cells. Other bacteria export toxins into the extracellular environment prior to binding to receptors on the plasma membrane of sensitive cells. At the plasma membrane, toxins are mediated by clathrin-mediated endocytosis, nonclathrin-mediated endocytosis, or caveolae-mediated endocytosis. Subsequent to entry into cells, toxins enter sorting compartments prior to being trafficked to endosomes. Some toxins are translocated within the acidic environment of endosomal vesicles, whereas others are trafficked through the Golgi apparatus to the endoplasmic reticulum (ER), where the A fragments are believed to be translocated through existing secretion pores into the cytosol.

this signal transduction modulation is the increase of cellular cyclic guanosine monophosphate (cGMP), which activates a cGMP-dependent protein kinase (PKG-II) and leads to the phosphorylation of a number of proteins involved in ion transport across the intestinal membrane (Giannella and Mann, 2003).

Receptor binding of intracellular-acting AB toxins, which do not remain at the cell surface but are internalized into the cell, can also result in the modulation of signal transduction pathways. In this manner, AB toxins can elaborate multiple effects on sensitive cells. Examples include cholera toxin, *E. coli* heat-labile toxin, and the Shiga and Shiga-like toxins. Most of the Shiga toxins bind to glycolipid Gb3. Interestingly, the toxin-induced production of cytokines, such as interleukin-1 beta and tumor necrosis factor upregulates the synthesis of Gb3, which facilitates further cellular intoxication (Sandvig, 2001).

7.1.2.3. Receptor Binding Can "Cross-link" Host Cells

Another important class of toxins whose members act by binding to cell-surface receptors are the bacterial superantigens. Superantigens produced by *S. aureus* and *S. pyogenes* bind to two receptors simultaneously, the major histocompatibility complex (MHC) class II molecules expressed on the surface of antigen-presenting cells, and the variable part of the T cell receptor (Vβ or Vγ) outside the antigen-binding groove. By simultaneously binding both receptors, superantigens cross-link the two immune cells, which activates T cells in a nonspecific manner, leading to massive T cell proliferation and the overproduction of proinflammatory cytokines such as interleukin-1 beta, tumor necrosis-factor alpha, and interferon gamma (Muller-Alouf et al., 2001). The consequences of activating signal transduction pathways by superantigens are described later in this chapter.

7.1.2.4. Receptor Binding Can Promote Membrane Insertion

A number of pore-forming toxins bind to receptors prior to assembling into higher-ordered complexes and inserting into the host cell membrane. Why would this be? Why not simply "land" on the plasma membrane and insert into the lipid bilayer? While the answers to these questions continue to be investigated, receptor binding may orient toxin monomers so that they can readily find one another on the rugged landscape of the mammalian plasma membrane for assembly into a higher-ordered complex. Receptor binding might also properly orient the assembled toxin complex for insertion into the membrane. However, these models for receptor function are not entirely satisfactory, because a number of pore-forming toxins can insert into artificial membranes and form pores, even in the absence of the receptor. Receptor-binding may, in the end, act to increase the effectiveness of pore-forming toxins, which may be important if the toxin is present at low concentrations in vivo. Examples of bacterial pore-forming toxins that bind to host cell receptors include the aerolysins secreted by *A. hydrophila* and other *Aeromonas* spp. (Abrami et al., 2001).

Notably, several intracellular-acting AB toxins also insert into target cell membranes to form ion-conducting channels. As we shall discuss later, the formation of transmembrane pore structures by the AB toxins may be important for the translocation of A fragments into the cytosol of mammalian cells. Recall that AB toxins bind to specific plasma membrane receptors, and it is thus possible that the receptors might participate in the assembly of toxin monomers into a higher-ordered complex and insertion into the lipid bilayer. However, again, the fact that the B fragments of diphtheria, anthrax, and botulinum toxins can insert into the lipid bilayer and form channels in artificial membrane systems lacking their normal protein receptors suggests that while not essential for the pore-forming activities of AB toxins, receptor binding may contribute to the "efficiency" of membrane insertion and/or channel formation by these toxins.

7.2. *Portals and Pathways: Entry of Intracellular-acting Toxins into Cells*

While some toxins are capable of modulating host cells at the plasma membrane, many other toxins must enter target cells to elaborate their cytotoxic effects. The requirement for this seemingly more "complex" mode of toxin action stems from the fact that many important toxin targets are only accessible from within the intracellular cytosolic compartment. Recall that the "active" moieties of most intracellular-acting toxins are enzymes. The transport of soluble enzymatic fragments across the membrane barrier of the host cell is a complex problem that needs to be addressed for toxins to be effective. There are three overall mechanisms by which intracellular-acting bacterial toxins are able to solve this problem: (1) direct injection from the bacterial cell into the host cell cytosol; (2) transport of a toxin's enzymatic fragment directly across the plasma membrane; or (3) transport of a toxin's enzymatic moiety from an intracellular location following receptor-mediated endocytosis of the entire toxin (Figure 11).

7.2.1. Direct Injection from the Bacterial Cell into the Host Cell Cytosol

As previously described, type III and, in some cases, type IV secretion mechanisms allow Gram-negative bacteria to modulate host cells by injecting effector proteins into cells with which they have formed an intimate relationship. These mechanisms represent extremely efficient modes of toxin delivery, as the toxin effectors themselves neither are trafficked within the host's endocytic pathways nor need to translocate themselves across the plasma membrane barrier. Instead, the injection needles, which are either flagellar-like (type III) or pilin-like (type IV) channels spanning the bacterial and eukaryotic membranes, are assembled to promote the safe passage of toxin effectors into host cells (Figure 11). Thus, these toxins must neither escape the harsh environment of the extracellular matrix nor bind plasma membrane receptors to enter cells. Many type III toxin effectors are aided in their intracellular journey by bacterial-derived chaperones that act, in part, to maintain the toxin in a "transport-competent" state. Despite the seemingly simple elegance of these direct-injection systems, much remains to be learned about the release, folding, and/or processing of the effectors upon exiting the injectosome into the cytosol of target cells.

7.2.2. Transport Directly Across the Plasma Membrane into the Cytosol

The passage of soluble protein toxins directly across the plasma membrane into the cell is thermodynamically unfavorable. Nonetheless, direct transport across the plasma membrane has been found for the adenylate cyclase toxins, produced by several species of the genus *Bordetella* (Figure 11). Analogous to the intracellular-acting AB toxins, a discrete fragment of the *B. pertussis* adenylate cyclase toxin binds the toxin to a specific cellular receptor,

CD11b/CD18, a member of the β2 integrin family (Guermonprez et al., 2001). In addition, a fragment of the toxin forms small, ion-selective channels in target cells, a characteristic of the B-domain of some (but not all) of the AB toxins related to the capacity to translocate an A fragment into the cytosol of target cells. Despite these similarities to the AB toxins, neither receptor binding nor channel formation may be absolutely essential for the membrane translocation of the adenylate cyclase toxin enzymatic fragment. Instead, the plasma membrane potential and the electrostatic charge of the catalytic domain appear to be the driving forces for transport of this catalytic fragment into the cell (Zaretzky et al., 2003), although many mechanistic details of this process remain unresolved. There is considerable interest in understanding the entry mechanisms of the adenylate cyclase toxins, with the goal of having the capability to someday transport peptide and protein-based therapeutics directly across the plasma membrane into target cells.

7.2.3. Transport into the Cell from an Intracellular Vesicle into the Cytosol

Translocation of most AB toxin enzymatic fragments requires that the toxins first (1) enter the cell, and (2) be trafficked to the appropriate portal for entry into the cytosol. Bacterial AB toxins satisfy these requirements by hijacking existing cellular entry and transport pathways (Figure 11). Endocytic pathways serve as the "subway systems" for toxins to travel into the depths of the cell. In these pathways, membrane-bound vesicles serve as subway "cars" that travel along "railways" (e.g., microtubules) between different "stations" (e.g., cellular organelles). Entry into the correct "rail-line" has a direct bearing on both the toxin's final destination and the efficiency with which the toxin ultimately reaches its molecular target.

7.2.3.1. Clathrin-dependent Endocytosis

Certain bacterial toxins enter target cells by clathrin-dependent endocytosis, which is the major sorting mechanism utilized by eukaryotic cells for efficient uptake of proteins and lipids from the cell surface (Mousavi et al., 2003). At the plasma membrane, toxin/receptor complexes concentrate in membrane "pits," which invaginate to form vesicles coated with the protein clathrin. Coated vesicles bud from the inner plasma membrane and rapidly lose their coats to form free vesicles. These toxin/receptor-containing vesicles are transported into the cell where they fuse with intracellular compartments, collectively termed early endosomes. Upon reaching the endosome, toxins are either translocated into the cytosol or further trafficked to an alternative cytosolic portal. Toxins that enter through clathrin-dependent pathways include diphtheria toxin, *Pseudomonas* exotoxin A, Shiga toxin, and cholera toxin (sometimes) (Sandvig, 2003). Interestingly, when the clathrin-dependent pathway is blocked, Shiga toxins can still enter the host cell cytosol, albeit less efficiently, suggesting that some toxins can utilize

alternative cellular entry mechanisms that are independent of clathrin-coated vesicles (Sandvig, 2003).

7.2.3.2. Clathrin-independent Endocytosis: Caveolae-mediated Uptake

Alternative pathways of toxins uptake into cells not involving clathrin-coated vesicles have been discovered, and are collectively called "clathrin-independent" endocytic pathways, although the specific features of each of these pathways may be very different (Nichols and Lippincott-Schwartz, 2001). One such clathrin-independent mechanism is called caveolae-induced endocytosis (Nichols and Lippincott-Schwartz, 2001), which involves the formation of flask-shaped membrane invaginations within the plasma membrane called "caveolae." Several toxins, including cholera and tetanus toxins, enter caveolae containing their receptors to be taken into the cell. Caveolae have unique molecular features relative to the rest of the plasma membrane, including a high cholesterol and ganglioside content, as well as associated proteins (including caveolin). Although caveolae-induced endocytosis shares some overall similarities with other vesicular trafficking events including vesicle budding, docking, and fusion, the mechanism by which caveolae mediates uptake of toxins and other molecules has been shown to have some unique features, which may provide an alternative entry pathway for some toxins (Nichols and Lippincott-Schwartz, 2001; Sandvig, 2003). For example, caveolae-mediated trafficking may allow some toxins to access novel intracellular compartments, as well as to bypass cellular degradation mechanisms.

7.2.3.3. The Role of Lipid Rafts in Toxin Entry

To successfully modulate cells, toxins bind their receptors at the correct location on the plasma membrane to ensure uptake. But how do receptors "find" the correct location for cellular entry? An emerging model that might help to explain this phenomenon is that many toxins encounter their cognate receptors within specialized microdomains within the plasma membrane called lipid rafts. Lipid rafts are dynamic, detergent-resistant microdomains that are enriched in cholesterol, glycosphingolipids, GPI-anchored proteins, and receptors, and have properties that are distinct from the "bulk membrane" (Brown and London, 1998). Among other things, lipid rafts are believed to function as concentrating platforms, where toxins (and other ligands) can bind to their cognate receptors, which are present in high concentrations. The clustering of the anthrax toxin B fragment receptor within lipid rafts triggers efficient internalization of the toxin by a clathrin-dependent process (Abrami et al., 2003). Multiple subclasses of lipid rafts, with different molecular compositions, function in either clathrin-dependent or -independent mechanisms of toxin entry into cells. Markers of lipid rafts are frequently found in caveolae, suggesting a role of lipid rafts in these nonclathrin-sorting pathways (Nichols and Lippincott-Schwartz, 2001). It is tempting to speculate that individual lipid raft subclasses may be linked to different vesicle-mediated

entry pathways. However, the extent to which the preferential binding of a toxin to a component of a particular membrane microdomain may drive, in part, the pathway of cell entry remains to be seen. Recently, the idea has emerged that the capacity of a specific toxin to engage multiple mechanisms of cell entry may increase the chances of successful intoxication. For instance, cholera toxin seems to be internalized through multiple endocytic mechanisms, including clathrin-dependent endocytosis and caveolae-mediated uptake (Orlandi and Fishman, 1998).

7.3. Portals for Toxin Entry into the Cytosol

Having entered mammalian cells by one of several endocytic mechanisms, toxins must be trafficked to the appropriate intracellular portal for delivery of A fragments into the cytosol. To be successful in their journey, toxins must escape possible degradation at the terminal end of several endocytic pathways. Passing through the entry portal into the cytosol occurs by two divergent overall strategies. One strategy exploits the acidic environment of the endocytic pathway, while the other makes use of the cellular secretion pathway, but only in reverse (Figure 11).

7.3.1. Exploiting the Acidic Environment of Endosomal Compartments

The use of existing endocytic pathways may seem a clever strategy for toxins to enter cells, but such a mechanism, as we have previously mentioned, can be wrought with peril. Some proteins ultimately are destined for degradation within the lysosome so that the cells can utilize the resulting breakdown products. To be effective, toxins must, at least to some degree, escape the consequences of encountering the degradative environment of the lysosome.

Some toxins, including diphtheria, anthrax, and the botulinum neurotoxins, escape the endocytic pathway prior to reaching the lysosome by exploiting the increasingly acidic conditions as the endocytic vesicles progress from the cell surface towards the center of the target cell along microtubules (Sandvig, 2003). In the case of diphtheria toxin, the receptor-bound protein is endocytosed and trafficked to early endosomal compartments, which lower their own pH by the action of membrane-associated, proton-pumping ATPases (Figure 11). The acidic pH of the endosomal compartments appears to be the critical feature that drives translocation of diphtheria toxin (as well as the catalytic fragments of anthrax and botulinum toxins into the cytosol). As the pH drops to approximately 5.0–6.0, these toxins undergo critical conformational changes that lead to the partial unfolding of the toxin structures and insertion of the translocation domains into the endosomal membrane. These events strongly correlate with the transport of the partially unfolded A fragments into the cytosol.

To date, the molecular details of toxin translocation are, in general, poorly understood. One common feature of bacterial toxins that are translocated

across endosomal membranes directly into the cytosol is the generation of toxin-derived, ion-selective channels in membranes upon exposure to acidic pH conditions (Nichols and Lippincott-Schwartz, 2001). Although toxin channels formed from the B fragments of AB toxins could conceptually function as water-soluble conduits for A fragment passage through the membrane, the exact mechanistic role of ion channels in toxin transport remains unresolved, despite intensive study.

A possible role for host cell proteins in toxin translocation has only recently been revealed. A host cell cytosolic translocation factor (CTF) complex facilitates the in vitro translocation of the diphtheria toxin catalytic domain from purified early endosomes to the external medium (Ratts et al., 2003), suggesting that the toxin alone may not be entirely sufficient for efficient translocation to occur. In addition to this cytosolic factor, diphtheria toxin's receptor may also contribute to the translocation process, as changes in the diphtheria toxin receptor binding results in low translocation efficiency (Saelinger, 2003).

7.3.2. Exploiting the Cell's Secretion Machinery

A second overall strategy used by bacterial toxins to access cytosolic portals is to move upstream within the pathway normally used by mammalian cells to secrete proteins (Sandvig, 2003). Newly synthesized nascent proteins destined for secretion are transported from the endoplasmic reticulum (ER), via the Golgi stack and the Trans Golgi network (TGN), to secretory vesicles that ultimately fuse with the plasma membrane to release the proteins into the extracellular environment. The secretion pathway is at least partially reversible, for example, when resident ER proteins need to be retrieved from the Golgi to the ER. Likewise, it seems that bacterial protein toxins can sometimes use a similar "retrograde" pathway to be trafficked from the plasma membrane to the ER lumen, where the A fragments are transported into the cytosol through what appears to be preexisting protein pores located within the ER membrane (Figure 11). Retrograde trafficking has been demonstrated for several toxins, including cholera toxin, Shiga toxin, and *P. aeruginosa* exotoxin A.

7.3.2.1. Toxin Trafficking from the Endosomal Pathway to the Golgi Apparatus.

Cholera toxin was the first bacterial toxin demonstrated to be transported from an endosomal pathway to the Golgi apparatus, but this same overall mechanism is now known to be used also by Shiga and pertussis toxins (Sandvig, 2003). Although cholera toxin can enter cells using multiple uptake pathways, only a nonclathrin-mediated pathway results in trafficking to the Golgi apparatus. Routing to the Golgi apparatus is complex, and seems to occur by several overall pathways. One pathway involves transport from the early endosomes to the Golgi via the late endosomes. In contrast, Shiga toxin is transported to the Golgi apparatus directly from the early endosomes.

Both the uptake and trafficking of toxins within targeted cells are highly regulated processes. One common feature of endosome to Golgi transport is the importance of a protein named dynamin, a member of the GTPase protein family that is essential for many intracellular membrane trafficking events. Since dynamin is required for both clathrin-dependent and clathrin-independent endocytosis, it is not surprising that this protein may also play a role in both clathrin-dependent and clathrin-independent routing within the cell. Endosome to Golgi transport is also regulated by cellular signaling, involving both protein kinases A and C. The association of toxin/receptor complexes with lipid rafts as well as the actual lipid composition of the rafts are important for toxin sorting as well (Sandvig, 2003; Sandvig and van Deurs, 2002). Cholesterol not only is crucial for endocytosis but also plays a role in regulating endosome to Golgi transport of toxins such as Shiga toxin and cholera toxin.

7.3.2.2. Toxin Trafficking from the Golgi to the ER

Trafficking of toxins to the ER is not only dependent on the receptor and membrane vesicle classes used to enter cells but also some toxins themselves possess ER localization signals. Normally, proteins trafficked from the Golgi to the ER via the retrograde pathway possess a carboxyl-terminal signal designated by the amino acids lysine-aspartate-glutamate-leucine (K-D-E-L, using the single letter designation for amino acids). The "KDEL" motif of ER resident proteins functions as an ER retrieval signal, which binds to so-called KDEL receptors found throughout the Golgi apparatus and the TGN. The ligand/KDEL receptor complex is transported in retrograde fashion in coat protein I (COPI)-coated vesicles. Interestingly, a number of toxins destined for the ER as a portal of entry into the cytosol also possess KDEL, or KDEL-like, localization signals. The cholera toxin and *E. coli* heat-labile A fragments contain KDEL and RDEL (a related sequence), respectively. *Pseudomonas* exotoxin A has the sequence R-D-E-L-K, and the Lys residue (K) is removed by a carboxyl-peptidase during the intoxication process. Notably, ER localization mechanisms exist independent of the involvement of KDEL receptors, as Shiga toxin, Shiga-like toxins, and the cholera toxin B fragment, although lacking a KDEL or a KDEL-like sequence on the translocated polypeptide, can still move in a retrograde direction toward the ER (Sandvig, 2003; Sandvig and van Deurs, 2002).

7.3.2.3. Translocation out of the ER

Once in the ER, the catalytic fragments of AB toxins still must traverse the ER membrane to access their cytosolic targets. Although much remains to be learned about the mechanism of toxin translocation out of the ER, there is mounting evidence of the involvement of a preexisting membrane channel complex whose central component is the Sec61 protein. The Sec61 complex normally transports misfolded proteins within the ER lumen into the cytosol

for subsequent degradation by the proteasomes. Since *Pseudomonas* exotoxin A and cholera toxin have both been found to associate with this complex, it is possible that the Sec61 complex may also act as a toxin translocator (Sandvig, 2003; Sandvig and van Deurs, 2002).

When toxins reach the ER lumen, protein disulfide isomerase catalyzes the dissociation of the disulfide-linked A and B fragments, which may result in the partial unfolding of the A fragments and exposure of hydrophobic regions. The Sec61 complex has been proposed to mistakenly recognize partially unfolded A polypeptides as "misfolded" proteins, and then translocate them across the ER membrane to the cytosol (Lord et al., 1999). The mechanisms by which the translocated polypeptides escape the degradation pathway in the cytosol and refold into their correct conformation to exert their enzymatic activities remain to be clarified. One intriguing hypothesis is that these toxin A fragments are not properly ubiquinated, because of their unusually low content of lysine residues that are normally targeted for modification by ubiquitin ligase (Rodighiero et al., 2002). The lack of ubiquination is significant because this posttranslational modification normally targets proteins to the proteosome for degradation.

7.4. Some Bacterial Toxins Cross Mucosal Barriers by the Process of Transcytosis

Some pathogens use toxins to modulate host cells in the tissues underlying the mucosal barrier. For those extracellular pathogens that do not traverse or disrupt the epithelial barrier, toxin effectiveness may depend on the ability to access target cells or molecules on the opposite side of the membrane. Bacterial toxins sometimes accomplish this using a host cellular trafficking mechanism called transcytosis (Dickinson and Lencer, 2003). Transcytosis allows multicellular organisms to selectively move material between two environments on opposite sides of membranes by the uptake and release of molecules from coated vesicles without disturbing the integrity of the epithelial barrier. Transcytosed molecules bind to specific membrane receptors, which direct the proteins into and across the epithelial cell by vesicular trafficking. The polymeric immunoglobulin receptor (pIgR) and the neonatal Fcγ receptor (FcRn) are examples of receptors that target their bound ligands to the transcytosis pathway (Dickinson and Lencer, 2003).

Bacterial toxins such as cholera toxin, Shiga-like toxin, and botulinum toxin can move across the epithelial barrier by transcytosis. Bacterial toxins that have traversed the epithelial barrier by transcytosis are presumably capable of interacting with other cells underneath the epithelial barrier they normally could not access, including antigen-presenting cells and other cells linked to the innate or adaptive immune response. However, these toxins use a fundamentally different mechanism (the indirect pathway) than that used by immunoglobulins (the classical pathway). Recall that after binding to GM1, cholera toxin is trafficked to the Golgi apparatus and then the ER. It

is from these organelles that cholera toxin apparently enters the transcytosis pathway and moves to the basolateral membrane via the mechanism responsible for sorting of nascent apical and basolateral membrane proteins. Unlike the "classical pathway" of transcytosis in which sorting motifs are embedded within the protein structure of the toxin, transcytosis of cholera toxin is driven by the lipid-based membrane anchor presented by the toxin's GM1 receptor, which concentrates the toxin in lipid rafts present within polarized human intestinal epithelial cells (Dickinson and Lencer, 2003). Lipid-based sorting motifs may also direct the transcytosis of other toxins that bind to lipid-based receptors.

7.5. Gram-positive Large Pore-forming Toxins: A Novel Mechanism for the Delivery of Virulence Factors into the Cytosol of Target Cells?

As discussed earlier, Gram-negative bacterial pathogens use several powerful and efficient transport systems to directly inject effector toxins into the cytosol of host target cells. It would perhaps be surprising if Gram-positive bacteria did not possess similar protein delivery mechanisms. Recently, a scheme for transporting Gram-positive effectors into target cells, called cytolysin-mediated translocation, has been proposed to be the functional equivalent of type III secretion in some Gram-negative bacteria (Madden et al., 2001).

Interestingly, cytolysin-mediated translocation is believed to be mediated by the action of a group of highly conserved toxins called cholesterol-dependent cytolysins (CDC), which are produced by pathogens within the genera *Streptococcus*, *Clostridium*, *Listeria*, and *Bacillus*. Monomers of secreted CDCs assemble into large transmembrane holes or pores approximately 30 nm in diameter within the plasma membrane of eukaryotic cells, which may function as conduits for the delivery of intracellular toxin effectors into host cells. The paradigm for this model is *S. pyogenes*. This pathogen secretes a factor (Spn) into the extracellular medium that possesses NAD-glycohydrolase activity. Although Spn elaborates cytotoxicity from within host cells, the mechanism of cellular entry is initially elusive, as the toxin appears to lack the necessary B fragment for translocation into the cell. Recently, evidence has emerged that Spn may, in fact, be translocated into mammalian cells through streptolysin O–generated pores (Madden et al., 2001). Streptolysin O (and other CDCs) are increasingly used by cell biologists to introduce large molecules into eukaryotic cells, further suggesting the idea that these large, pore-forming toxins could be used by Gram-positive pathogens to deliver virulence factors into host cells (Walev et al., 2001). While a great deal remains to be learned about toxin-based transport of other toxin effectors, the identification of cytolysin-mediated translocation suggests an additional function for toxins that had not been previously recog-

nized, and is an exciting initial step toward understanding the possible role of large pore-forming toxins produced by Gram-positive bacteria.

8. Modulation of Target Cell Function

Subsequent to synthesis, export from bacteria, and establishing appropriate interactions with target cells, toxins are poised to carry out the purpose for which they were generated: to modulate host cell properties. The diversity of strategies required by pathogenic bacteria to be successful in different host niches is reflected, in part, by the variety of bacterial toxins that are used for remodeling the host. Toxins exert a biochemical activity that affects the function of a primary target, which can either be a specific eukaryotic molecule or, alternatively, a macromolecular complex such as the membrane. The alterations of primary targets by toxins are rarely isolated occurrences, but instead can sometimes be felt throughout the cell. For example, targeting an integral component of a signaling pathway will modulate all the downstream cellular functions regulated by that particular pathway. Thus, the primary effects of toxins can be amplified in such a manner as to alter cellular properties in several quantifiable ways. These cellular changes can further result in profound "global" consequences within the host. For example, modulating the tight junctions that bond together epithelial cells could alter the manner in which epithelial cells interact, thereby potentially allowing pathogenic bacteria to breach epithelial barriers to reach the underlying tissues. A second illustration is the manner in which toxin-mediated modulation of cells involved in innate or adaptive immunity could profoundly affect the capability of the host to effectively respond to invading pathogens, which would significantly impact the outcomes of bacteria-mediated diseases.

An important reccurring principle stated several times throughout this chapter is that, in general, we have a poor understanding of the exact roles of toxins in bacterial pathogenesis. Why is this? In the context of our current discussion, one explanation may be the multiple cellular and global consequences of toxin action. For example, the *H. pylori* vacuolating cytotoxin (VacA) causes multiple effects on mammalian cells, including redistribution of late endocytic compartments, increased mitochondrial membrane permeability, activation of the p38/ATF-2 signal transduction pathway, apoptosis, and inhibition of T cell activation. This wide range of cellular effects can be attributed to the interactions of VacA with multiple cell surface receptors and intracellular localization of the toxin to both endosomes and mitochondria. The difficulty lies in differentiating which of these consequences contributes directly to the virulence strategies of the pathogen, and which (if any) must be accepted as "collateral" consequences.

Understanding the role(s) of any particular toxin in pathogenesis is best understood by adopting a multilayered approach. That is to say, we must use the tools of biochemistry, molecular biology, and structural biology to

identify the molecular basis of toxin action. At that point, cell biology and systems biology approaches are required to understand the global consequences of toxin action to the intoxicated cell. Finally, genetics, coupled with appropriate animal models and epidemiological observations are needed to appropriately unravel consequences of the toxin to the host. Only by studying toxins in such a holistic manner can we begin to adequately comprehend the role of most toxins in pathogenesis.

We shall now examine major paradigms for toxin action. The known biochemical functions of extracellular- and intracellular-acting toxins will be discussed. In addition, we will identify those eukaryotic targets known to be modified, as well as alterations in cellular and host properties that occur as a direct consequence of toxin action.

8.1. Extracellular-acting Toxins

Extracellular-acting bacterial toxins elaborate their modulatory effects at the surface of target cells. Extracellular-acting toxins can be classified as those that (1) modulate host cell signal transduction by binding to surface receptors, (2) alter host cell permeability by inserting into the plasma membrane to form pores, or (3) directly remodel the host cell surface by degrading components at, or near, the plasma membrane (Table 4).

8.1.1. Extracellular-acting Toxins that Modulate Signal Transduction Within the Host Cell by Binding a Plasma Membrane Receptor

Conceptually, one of the most straightforward mechanisms for pathogenic bacteria to modulate host cells would be to produce toxins that could interfere with eukaryotic signal transduction. Host cells sense their dynamic environment by cell-surface receptor binding of extracellular molecules that act as signals. The signal is transduced across the membrane and into the nucleus where specific genes are expressed in response. Recall that two prominent classes of toxins that modulate host signal transduction pathways by binding cell-surface receptors are the superantigens and the B fragments of the AB intracellular-acting toxins (Table 4).

8.1.1.1. Superantigens

Some toxins induce an exaggerated cell-mediated response resulting in symptoms that resemble those associated with shock. These toxins are called "superantigens," because they induce inappropriate polyclonal lymphocyte proliferation (Proft and Fraser, 2003). Recall that superantigens act as "cellular cross-linkers," by simultaneously binding two different receptors on two different cells, thereby bringing together antigen-presenting cells and T helper cells (Th cells). Antigen-presenting cells such as macrophages degrade protein antigens and display some of the resulting peptide epitopes in a complex with the MHC class II molecule on the cell surface. Normally, only a few

TABLE 4. Bacterial extracellular-acting toxins.

Category	Toxin	Activity	Molecular targets	Consequence
Superantigens	SEA-SEQ, TSST-1, SPEA, SPEC, SSA (*Staphylococcus aureus*)	Lymphocyte-transforming	MHC class II molecules, T cell receptor	T cell activation and cytokine secretion
Superantigens	SpeB (*Streptococcus pyogenes)*	Cysteine protease	Multiple host proteins, IgG, IgA, IgD, IgE and IgM	Alteration in immunoglobulin binding properties, apoptosis
Superantigens	IdeS (*Streptococcus pyogenes)*	Cysteine protease	IgG	Interfere with Fc-mediated phagocytic killing
Binding domain of some AB toxins	Pertussis toxin B fragments (*Bordetella pertussis*)	Receptor binding	Cell surface receptors	Induction of chemotaxis and cell proliferation
Binding domain of some AB toxins	Cholera toxin B fragments (*Vibrio cholerae*)	Receptor binding	Membrane receptor: ganglioside G_{M1}	Induction of cAMP-dependent Cl-secretory response
Binding domain of some AB toxins	Heat-labile toxin B fragments (*Escherichia coli*)	Receptor binding	Membrane receptor: ganglioside G_{M1}	Modulate the immune response
Pore-forming toxins	Streptolysin O (*Streptococcus*)	Cell membrane permeabilization	Cholesterol	Cell death, extracellular calcium influx into the cytosol
Pore-forming toxins	Perfringolysin O (*Clostridium perfringens*)	Cell membrane permeabilization	Cholesterol	Cell death
Pore-forming toxins	Leukotoxins (several pathogens)	Cell membrane permeabilization		Cell death
Pore-forming toxins	Aerolysin (*Aeromonas hydrophila*)	Cell membrane permeabilization	Glycophorin	Cell death, active Cl(-) secretion in the intestinal epithelium, possibly by channel insertion into the apical membrane and by activation of protein kinase C
pore-forming toxins	Delta hemolysin (*Staphylococcus aureus*)	Cell membrane permeabilization		Cell death
Pore-forming toxins	Hemolysin (*Escherichia coli*)	Cell membrane permeabilization		Cell death

(*Continued*)

TABLE 4. Bacterial extracellular-acting toxins.—Cont'd

Category	Toxin	Activity	Molecular targets	Consequence
Surface-acting toxins	Enterotoxin (*Bacteroides fragilis*)	Zinc-dependent metalloprotease	E-cadherin	Alteration of epithelial permeability and morphology, stimulates interleukin-8 (IL-8) secretion by human intestinal epithelial cells, IL-8 secretion involves tyrosine kinase-dependent activation of nuclear factor-kappa B (NF-kappa B) as well as activation of the mitogen-activated protein kinases (MAPKs), p38 and extra cellular signal-related kinase, which can be related to intestinal inflammation
Surface-acting toxins	Heat-stable toxins (*Escherichia coli*)	Receptor binding	ST receptor: guanylyl cyclase C	Increase of cellular cyclic (cGMP), activation of two protein kinases, protein kinase G (PKG), and protein kinase A (PKA), leading to phosphorylation of a chloride ion-channel, leading to efflux of chloride and water
Surface-acting toxins	Alpha-toxin (*Clostridium perfringens*)	Phospholipase C	Plasma membrane	Cell death due to influx and efflux of small molecules and ions

Th cells have receptors that will recognize this particular MHC peptide complex, resulting in the selective simulation of only those Th cells. In contrast, superantigens indiscriminately bind the MHC class II molecule and T cell receptors on many macrophages and T cells, respectively, even in the absence of the specific complexed antigenic peptide. Thus, instead of a macrophage stimulating 1 in 10,000 T cells (the normal response to an antigen), as many as 1 in 5 T cells can be stimulated by the binding of superantigens. When Th cells are stimulated by macrophages, one result is that T cells release cytokines, especially IL-2. However, the action of superantigens causes excessively high levels of IL-2 to circulate in the bloodstream, giving rise to T cell proliferation and a variety of symptoms, including nausea, vomiting, malaise, and fever (Muller-Alouf et al., 2001; Proft and Fraser, 2003). In addition to functioning as potent T cell mitogens, bacterial superantigens can sometimes also modulate the properties of B lymphocytes and cells of the myeloid lineage, including antigen-presenting cells and phagocytes.

Because of their ability to subvert the normal adaptive immune responses, superantigens may be involved in immune-related disorders. For example, superantigens may contribute to autoimmune diseases by activating T cells that are specific for self-antigens (Proft and Fraser, 2003). Moreover, superantigens and other bacterial effectors sometimes act synergistically to cause maximal toxic effects. For example, a combination of lipopolysaccharide (LPS) and staphylococcal enterotoxin B induces a bimodal pattern of hepatic dysfunction within animals, leading to late liver injury, whereas animals treated with either agent alone do not develop the same pathology (Beno et al., 2003).

Approximately 40 bacterial superantigens have been described thus far (Muller-Alouf et al., 2001). The complete sequences of microbial genomes have accelerated the identification of bacterial factors that could potentially act as superantigens. The best-characterized superantigens are the staphylococcal enterotoxins (SEs) and the streptococcal pyrogenic exotoxins (SPEs) (Alouf and Muller-Alouf, 2003). The staphylococcal superantigens include 18 classic enterotoxins designated as SEA, SEB, SEC (and antigenic variants), D, E, and the newly discovered enterotoxins SEG to SEQ. These SEs are the causative agent of staphylococcal food poisoning and may induce diarrhea and vomiting. In addition, TSST as well as the exfoliating toxins A and B also belong to the staphylococcal superantigen family. There are 12 superantigens produced by Group A streptococci, most of which are associated with *S. pyogenes*. These superantigens include pyrogenic exotoxins designated as SPEA and SPEC, and the recently identified pyrogenic toxins, SPEG to SPEM, SMEZ (streptococcal mitogenic exotoxin), and SSA (streptococcal superantigen). These toxins are able to cause streptococcal toxic shock syndrome, which is the most severe form of invasive streptococcal disease. In addition to superantigens generated by streptococcal and staphylococcal species, superantigens are also produced by *Yersinia pseudotuberculosis*, *C. perfringens* and *Mycoplasma arthritidis*.

8.1.1.2. B Fragments of the AB Intracellularly Acting Toxins

The B fragments of AB intracellular-acting toxins can modulate the properties of eukaryotic cells in two separate ways: by first activating intracellular signaling through receptor binding, and then by facilitating the delivery of enzymatic A fragments into the cytosol. The B fragments of cholera and the *E. coli* heat-labile enterotoxins, Shiga toxin, and pertussis toxin all function as activators of signal transduction. In the case of heat-labile toxin, binding to ganglioside receptors on the surface of B lymphocytes can modulate the immune response by altering the expression of important cell surface molecules including class II MHC and CD25, which is the alpha-chain of the IL-2 receptor (Bone et al., 2002). The A fragments of Shiga toxin and Shiga-like toxins produced by *S. dysenteriae* serotype 1 and enterohemorrhagic *E. coli*, respectively, cause cell death by inhibiting protein synthesis. Interestingly, the B fragments alone are capable of inducing cell death by a distinct mechanism involving the activation of pathways by receptor binding leading to programmed cell death (apoptosis) in several different cell types (Katagiri et al., 1999; Mangeney et al., 1993). A final example is the pertussis toxin, whose B fragments induce signal transduction through the eukaryotic inositol phosphate pathway (Rappuoli and Pizza, 2000).

8.1.2. Extracellular-acting Toxins that Modulate Host Cells by Inserting into the Membrane to Form Pores

The pore-forming toxins act by inserting into the plasma membrane of target cells in such a manner as to form transmembrane pores or channels. Pore formation, in part, modulates the permeability barrier, which normally limits the passage of macromolecules as well as many smaller solutes into, and out of, target cells. In addition to altering the permeability barrier, the action of pore-forming toxins sometimes affects target cells in several other distinct ways, including the modulation of cytokine release, activation of intracellular proteases, and the induction of apoptosis (van der Goot, 2003).

8.1.2.1. How Do Pore-forming Toxins Insert into the Membrane?

The mechanism of pore formation has been studied using both artificial membrane systems such as liposomes and a variety of cell types. In general, pore-forming toxins are exported from bacteria as soluble monomeric proteins. A fundamental issue surrounding the action of pore-forming toxins is the mechanism by which these otherwise soluble proteins are able to insert into the membrane and form pores. One explanation is that pore-forming toxins can exist in two distinct states: a stable soluble state, which is converted to an insoluble, yet stable, state within the lipid bilayer. Toxin monomers associate with the plasma membrane of target cells in a manner that may be dependent, in part, on specific surface molecules, including cholesterol and the glycosylphosphatidyl (GPI)-anchored proteins. Bound toxin monomers

diffuse laterally within the two-dimensional landscape of the membrane until they encounter each other and undergo circular polymerization to form ring-shaped structures sometimes called pre-pores, which sit on top of the lipid bilayer. The ring-shaped complexes can vary in size, and comprise structures involving between approximately 6 and 50 monomers. The pre-pore structures undergo conformational changes that unmask and expose hydrophobic surfaces, promoting insertion into the lipid bilayer and the formation of transmembrane pores. The primary structures of toxins seem to dictate the entire transition, as no additional factors such as chaperones or transporters are required (van der Goot, 2003). Recall that transmembrane pores are generally built out of either α-helical or β-sheet elements that assemble in a manner such that polar and charged amino acids face inward into the lumen of the pore, while hydrophobic and aromatic amino acids face into the fatty acid core of the lipid bilayer. Although the pore diameters, in principle, dictate the size of molecules that can pass through the lumen, some toxin channels have gating mechanisms that establish a degree of selectivity to the components that can traverse the membrane (van der Goot, 2003).

8.1.2.2. Classes of Pore-forming Toxins

There are several classes of pore-forming toxins that differ broadly in sequences, pore sizes, and functional properties.

The "Large" Pore-forming Toxins. The large pore-forming toxins form holes within the membrane that range from 35 to 300 Å in diameter. These rather large conduits allow the passage of both small solutes and large macromolecules. Not surprisingly, higher concentrations of these toxins induce rapid cell death. However, lower concentrations do not kill cells, but modulate important signal transduction pathways (van der Goot, 2003). In addition, recall that some of the large pore-forming toxins have been proposed to facilitate the transport of additional virulence factors into host cells. These toxins are also called the cholesterol-binding toxins, because their functions are dependent on the presence of cholesterol in target membranes. Large pore-forming toxins are produced by a number of Gram-positive pathogens, including *S. pyogenes*, *S. pneumoniae*, *Bacillus* spp., Clostridial spp., and *Listeria*.

The "Small" Pore-forming Toxins. The small pore-forming toxins assemble into structures with relatively small pores or channels whose diameters range from 10 to 15 Å. Pores of this size are sufficient to allow the selective transport of solutes smaller than 2,000 Da in molecular weight, while large macromolecules are retained within the cell. Consequences of small pore formation include membrane rupture and cell lysis, leading to cell death (Rappuoli and Pizza). Examples of small pore-forming toxins include *S. aureus* α-toxin, leukocidin F, leukocidin S, and α-lysins A and C.

The Family of RTX Toxins. The repeats in toxin (RTX) toxins are a specific subset of the small pore-forming toxins. RTX toxins have several unique

characteristics that are not observed in other pore-forming toxins, including 10–40 repeats of a conserved glycine- and aspartate-rich motif comprising nine amino acids (XLXGGXG(N/D)D). These signature repeats are believed to comprise calcium-binding motifs, which are important for host cell association. The RTX structural gene is located within an operon of four genes that is conserved between different species (Ludwig and Goebel, 1999; Oxhamre and Richter-Dahlfors, 2003).

While several Gram-negative bacteria produce RTX toxins, the *E. coli* α-hemolysin is probably the most thoroughly characterized. α-hemolysin inserts into host cell membranes and forms transmembrane pores that are 1 nm in diameter. α-hemolysin pores are characteristically unstable, hydrophilic, and cation-selective. Individual RTX toxins differ significantly in their target cell specificities. However, the primary consequence of cellular intoxication by members of this toxin family is lysis due to osmotic imbalances caused by pore formation (Ludwig and Goebel, 1999; Oxhamre and Richter-Dahlfors, 2003). More recently, low concentrations of RTX toxins have been demonstrated to modulate intracellular signaling without causing cell lysis (Oxhamre and Richter-Dahlfors, 2003). This physiological effect, in fact, may be more relevant for disease processes because of the potentially low concentrations of toxin within the host.

The B Fragments of AB Intracellular-acting Toxins. The B fragments of several AB toxins are capable of inserting into lipid bilayers to form ion-conducting channels. Although these channels may resemble those of the small pore-forming toxins, several fundamental differences exist. While the small pore-forming toxins act at the plasma membrane of target cells, the B fragments of AB toxins are generally believed to form channels within membrane-bound vesicles that are either early or late endosomes. In addition, channels formed by B fragments are generally believed to facilitate translocation of enzymatic A fragments across the endosomal membrane into the cytosol, whereas there are no known protein translocation functions associated with other small pore-forming toxins. The B fragments of intracellular-acting toxins that form channels associated with membrane translocation include those of diphtheria toxin, anthrax toxin, and the botulinum neurotoxins.

8.1.3. Extracellular-acting Toxins that Directly Remodel the Host Cell Surface by Degrading Molecules on or near the Plasma Membrane

Some pathogenic bacteria produce toxins that degrade extracellular matrix proteins or plasma membrane components of host cells, which may contribute directly to the colonization or spread of the organism within the host. Several degradative factors produced by pathogenic bacteria elaborate enzymatic activities similar to those already generated by mammalian cells. How, then, can these factors be damaging to the host? The inappropriate expression of toxins at the wrong time or place (from the host's standpoint) is likely to be responsible for the contribution of these factors to pathogenesis. For example, eukaryotic cells

generate several sphingomyelinases, which convert sphingomyelin into ceramide and other products of sphingolipid hydrolysis that act as secondary messengers within eukaryotic cells. Ceramide, in particular, is linked to the induction of mitochondria-dependent apoptosis pathways within target cells, resulting in cell death. Several pathogens also generate membrane-disrupting toxins with sphingomyelinase activities, which, when expressed at the wrong time or place, could obviously have profound effects on the viability of target cells.

8.1.3.1. Bacterial Toxins that Degrade Membrane Lipids

Some membrane-disrupting toxins act to degrade membrane lipids. These toxins cleave off the charged head groups or alternative sites on membrane lipids, which acts to destabilize the lipid bilayer structure and cause cell lysis (Titball, 1999). A variety of human or animal pathogens have been reported to produce lipid-degrading factors. Specific examples include the *C. perfringens* α-toxin (a zinc-metallophospholipase), the *S. aureus* β-toxin (a sphingomyelinase), and the *H. pylori* phospholipase A.

8.1.3.2. Bacterial Toxins that Degrade Extracellular Host Proteins

A second class of extracellular, hydrolytic factors are metalloproteases that cleave proteins associated with the plasma membrane or extracellular matrix. One of the best-known examples is the *B. fragilis* enterotoxin (BFT). BFT is a zinc-binding metalloprotease that rapidly cleaves the extracellular domain of E-cadherin, which is a transmembrane glycoprotein responsible for calcium-dependent cell–cell adhesion in epithelial cells. BFT-mediated cleavage of E-cadherin results in the alteration of tight junctions between polarized intestinal epithelial cells and the reversible stimulation of chloride secretion (Rappuoli and Pizza, 2000). Other extracellular proteases include the *P. aeruginosa* and *S. epidermidis* elastases, which cleave the important connective tissue protein elastin, and the *C. histolyticum* collagenase, which degrades collagen (Rappuoli and Pizza, 2000).

8.2. Intracellular-acting Toxins

Rather than functioning at the plasma membrane of target cells, many bacterial toxins elaborate their effects from the interior of eukaryotic cells, which is characterized by complex networks of organelles, cytoskeletal assemblies, and a large number of potential functional and regulatory targets. By modifying one or more of these targets, toxins are capable of directly modulating important metabolic and/or regulatory processes within the cell. A list of intracellular-acting toxins is shown in Table 5.

8.2.1. Many Intracellular-acting Toxins Function as Enzymes

Intracellular-acting toxins generally elaborate an enzymatic activity from within eukaryotic target cells. How might the elaboration of a catalytic

TABLE 5. Bacterial intracellular-acting toxins.

Category	Host system–modulated	Toxin	Activity	Target	Consequence
AB toxin	Protein synthesis	Diphtheria toxin (*Corynebacterium diphtheriae*)	ADP-ribosylation	Elongation factor-2	Protein synthesis inhibition; cell death
AB toxin	Protein synthesis	*Pseudomonas aeruginosa* exotoxin A	ADP-ribosylation	Elongation factor-2	Protein synthesis inhibition; cell death
AB toxin	Protein synthesis	Shiga toxin, *Escherichia coli* shiga-like toxins	*N*-glycosidase	28S RNA	Inhibits protein synthesis by catalyzing the depurination of a single adenine residue from the 28S ribosomal RNA, thereby inhibiting elongation factor-1-dependent peptide elongation; cell death
AB toxin	Signal transduction	Pertussis toxin (*Bordetella pertussis*)	ADP-ribosylation	G_i	Inactivation of G_i stimulates adenylyl cyclase resulting in increased cAMP levels
AB toxin	Signal transduction	Cholera toxin (*Vibrio cholerae*)	ADP-ribosylation	G_s	Activates adenylate cyclase and increases intracellular cAMP levels
AB toxin	Signal transduction	*E. coli* heat-labile toxins (LTs)	ADP-ribosylation	G_s	Activates adenylate cyclase and increases cAMP
AB toxin	Signal transduction	Anthrax lethal toxin (*Bacillus anthracis*)	Zn-metalloprotease	Mitogen-activated protein kinase-kinases	Cell death; inhibit antigen presentation by dendritic cells; inhibit the production of proinflammatory cytokines
AB toxin	Signal transduction	Anthrax edema toxin (*B. anthracis*)	Calmodulin-dependent adenylate cyclase	ATP	cAMP increase, leading to an imbalance of water homeostasis; cell death

AB toxin	Signal transduction	Cytolethal distending toxin (multiple pathogens)	Nuclease	DNA	Cell cycle arrest at G2/M transition; apoptosis; inhibition of epithelial-cell proliferation and apoptosis, enabling the invasion of bacteria; inhibition of proliferation of cycling immune cells, resulting in local immunosuppression; and inhibition of the fibrotic response, which can wall off invading bacteria; NF-κB-dependent proinflammatory effect
AB toxin	Signal transduction	*Bordetella* adenylate cyclase toxins	Adenylate cyclase	ATP	cAMP increase; inhibits neutrophil functions, including phagocytosis, oxidative burst, and chemotaxis; induces apoptosis in macrophages both in vitro and in vivo
AB toxin	Signal transduction	*Clostridium perfringens* alpha-toxin	Phospholipase C	Membranes	Gas gangrene; massive degradation of Phospahtidyl choline and sphingomyelin in membranes; increased vascular permeability and edema; activation of PKC leading to O_2^- production; hemolysis in rabbit erythrocytes
AB toxin	Signal transduction, cytoskeleton	*C. difficile* toxins A and B	UDP-glucosyl-transferase	Rho, Rac, Cdc42	Actin depolymerization and cell rounding cell death; ATP levels diminish; reduction in mitochondrial membrane potential reactive oxygen species increase;

(*Continued*)

TABLE 5. Bacterial intracellular-acting toxins—Cont'd

Category	Host system–modulated	Toxin	Activity	Target	Consequence
AB toxin	Signal transduction, cytoskeleton	*E. coli* cytototoxic necrotizing factors (CNF1 and CNF2) *Bordetella* dermo necrotic toxin (DNT)	Deamidase or transglutaminase	Rho, Rac, Cdc42	Formation of filopodia, membrane ruffles, and a dense network of actin stress fibers, indicating an activation of Rho proteins; formation of multinucleated cells
AB toxin	Signal transduction, cytoskeleton	*Pasteurella multocida* toxin		G alpha-q G alpha12/13	Activation of PLC; increased inositol phosphate production; stimulation of protein kinase C activity, Ca^{2+} mobilization, actin rearrange ments, and increased protein tyrosine phosphorylation
AB toxin	Cytoskeleton	*C. botulinum* C2	ADP-ribosyl-transferase	Monomeric G-actin	Actin polymerization failure
AB toxin	Vesicular trafficking	Tetanus toxin (*C. tetani*)	Zn-metalloprotease	VAMP/synapto-brevin	Spastic paralysis
AB toxin	Vesicular trafficking	Botulinum neurotoxins B, D, G F (*C. botulinum*)	Zn-metalloprotease	VAMP/synapto-brevin	Flaccid paralysis
AB toxin	Vesicular trafficking	Botulinum neurotoxins A, E(*C. botulinum*)	Zn-metalloprotease	SNAP-25	Flaccid paralysis
AB toxin	Vesicular trafficking	Botulinum neurotoxin C (*C. botulinum*)	Zn-metalloprotease	Syntaxin, SNAP-25	Flaccid paralysis
AB toxin	Vesicular trafficking, cellular viability	*H. pylori* vacuolating cytotoxin	Channel formation	Intracellular membranes, mitochondria	Vacuole formation; apoptosis; inhibits T-cell proliferation through a p53-dependent cell-cycle arrest in the G1 phase; localizes to mitochondria; causes mitochon-drial membrane permeabilization,

					characterized by membrane potential loss and cytochrome *c* release in cells
AB toxin	Cytoskeleton	*C. sordellii* lethal toxin	UDP-glucosyl-transferase	Rac, Ras, Ral	Induces cortical actin; depolymerization; localizes at mitochondria; induces cell death via disruption of mitochondrial homeostasis as assessed by an early decrease in mitochondrial membrane potential in isolated mitochondria
Type III secretion effector	Signal transduction, cytoskeleton	*Salmonella* SopE	Guanine nucleotide exchange factor	Cdc42	Membrane ruffling; cytoskeleton rearrangement; proinflammatory cytokine production; mediates bacterial internalization into host cell
Type III secretion effector	Cellular viability	*Salmonella* SipB		Caspase-1, mitochondria	Caspase-1-dependent macrophage cell death; autophagy induction
Type III secretion effector	Cellular viability, cytoskeleton	*E. coli* Map		Mitochondria	Disruption of mitochondria membrane potential; Cdc42-dependent filopodia formation; alters tight junction structure
Type III secretion effector	Signal transduction, cellular viability	*E. coli* EspF		Mitochondria	Disruption of host intestinal membrane barrier; cell death; induces mitochondrial membrane permeabilization; activates caspases 3 and 9

(*Continued*)

TABLE 5. Bacterial intracellular-acting toxins—Cont'd

Category	Host system–modulated	Toxin	Activity	Target	Consequence
Type III secretion effector	Cytoskeleton	*P. aeruginosa* ExoS, ExoT	N-terminal small G-protein activation (GAP) C-terminal ADP ribosyltransferase	Rho Ras	Alteration of signaling pathways Collapse of cytoskeleton
Type III secretion effector	Cytoskeleton	*P. aeruginosa* ExoU	Patatin-like phospholipase	Membrane lipids	Irreversible damage to cellular membranes and rapid necrotic death
Type III secretion effector	Signal transduction	*P. aeruginosa* ExoY	Adenylate cyclase	ATP	Actin disruption
Type III secretion effector	Cytoskeleton	*C. botulinum* C3	ADP-ribosyl-transferase	Rho A, B and C	Cellular actin stress fibers breakdown
Type III secretion effector	Cytoskeleton	*Salmonella* SopB	Inositol phosphate phosphatase	Inositol polyphosphates	Increase in chloride secretion; cytoskeleton rearrangement; antiapoptotic; causes a sustained activation of Akt/protein kinase B, a pro-survival kinase
Type III secretion effector	Cytoskeleton	*Yersinia* YopO/YpKA	Serine/threonine kinase	Binds to active or inactive Rho GTPase	Inhibition of phagocytosis; disrupts the actin cytoskeleton of cultured cells
Type III secretion effector	Cytoskeleton	*Yersinia* YopH	Tyrosine phosphatase	Fak,Cas, and paxillin in epithelial cells Cas, SKAP-HOM, Fyb, and the Fak-homolog Pyk in macrophages	Inhibition of phagocytosis; disruption of focal adhesions; apoptosis

Type III secretion effector	Cytoskeleton	YopE	G-protein activation	RhoA, Rac1, and Cdc42	Actin depolymerization; inhibit proinflammatory cytokine production
Type III secretion effector	Signal transduction	YopM	Adaptor function	PRK2, RSK1	Affects gene expression involved in cell cycle and cell growth; important for resistance to innate immunity
Type III secretion effector	Cytoskeleton	YopT	Cysteine protease	RhoA, Rac1, and Cdc42	Actin depolymerization; disruption of stress fibers, cell rounding, and inhibition of phagocytosis
Type III secretion effector	Cellular viability	YopP/YopJ	Cysteine protease	Target proteins with ubiquitin or a ubiquitin-like modification	Inhibition of proinflammatory responses; apoptosis; blocks activation of the MAPK kinases and IKK; suppression of cytokine production
Type III secretion effector	Cellular viability	*Shigella flexneri* IpaB	Adaptor function	Caspase I	Apoptosis
Type IV secretion effector	Signal transduction	*H. pylori* CagA	Adaptor function	Src homology (SH) 2–containing protein tyrosine phosphatase–2, Grb2, COOH-terminal Src kinase, hepatocyte growth factor receptor/c-Met, and zonula occludens–1	Activation of the tyrosine phosphatase SHP-2; cellular morphological changes (called the hummingbird phenotype); disruption of epithelial apical/junction complex; cytoskeleton rearrangement

activity facilitate the cellular modulatory activity of toxins? Analogous to the mechanisms by which many drugs act, could toxins not modulate the properties of their eukaryotic targets by simply binding to them? Although this may seem to be a reasonable strategy, the efficacy of toxins is dependent, in large part, on the delivery of effector domains, which are typically proteins of greater than 15,000 Da, at sufficient levels to induce cellular effects. As we have discussed, the road to cellular entry is complex and is, in fact, rather inefficient. Thus, the "effective" concentrations of intracellular toxins might conceivably never reach sufficient levels to bind and inactivate enough target molecules at any given time to alter cellular function. However, toxins with enzymatic activities can be potent even at extremely low concentrations because a single toxin molecule can catalyze the covalent modification of many intracellular target molecules. In fact, it has been estimated that a single molecule of the diphtheria toxin catalytic domain is sufficient to kill an entire cell (Yamaizumi et al., 1978).

8.2.2. How Bacterial Toxins Modulate Host Cells: Classes of Toxin Activities

We can classify intracellular-acting toxins as those that (1) act by modifying important molecular targets within mammalian cells, or (2) act by mimicking existing eukaryotic effectors. In this section, we will explore the activities elaborated by intracellular-acting toxins. Because the enzymatic activities are probably some of the most well-understood aspects of these toxins, we will group toxins here by the reactions they catalyze. However, knowing a toxin's enzymatic activity does not always provide clues as to the cellular outcome, i.e., a single enzymatic activity elaborated by two distinct toxins may result in two different sets of consequences within intoxicated cells.

8.2.3.1. Toxins that Modify Molecular Targets within Mammalian Cells

Toxins modify intracellular targets by catalyzing either (1) the addition of low–molecular weight chemical functional groups to the target molecules or alternatively (2) the "breakdown" of the target molecules into smaller fragments. In either case, the toxin-mediated modification of intracellular targets changes the capacity of these molecules to function properly.

Intracellular-acting Toxins that ADP-ribosylate Their Targets. The discovery that a discrete fragment of diphtheria toxin (the A fragment) is an enzyme with ADP-ribosyltransferase activity was a seminal event in our understanding of how bacterial toxins modulate target cells. To reiterate an important concept discussed above, the catalytic activities are responsible, in part, for the remarkable potency of most intracellular-acting toxins. The capacity of a single molecule of a toxin's A fragment to modify many target molecules has profound implications for the intoxicated cells.

Since the identification of the diphtheria toxin A fragment as an ADP-ribosyltransferase, the list of ADP-ribosylating toxins has steadily grown,

establishing the ADP-ribosylation of target molecules as one of the most prominent strategies by which pathogenic bacteria modulate eukaryotic cells. As a source of ADP-ribose, ADP-ribosylating toxins use NAD, which is perhaps better known as an enzyme cofactor for oxidation–reduction reactions. All ADP-ribosyltransferases catalyze the transfer of ADP-ribose to a specific amino acid on the target molecule, which functions as the ADP-ribose acceptor. Interestingly, there is a lack of overall amino acid homology between the different ADP-ribosylating toxins, and there is some evidence to suggest that the structure of the active site "scaffolds" may be most important for defining the characteristic features of all ADP-ribosylating toxins. Despite the lack of overall sequence conservation, extensive structure–function analyses of these enzymatic fragments have revealed the presence of an absolutely conserved active-site glutamic acid residue, which is now recognized as a hallmark of all ADP-ribosyltransferases (Barbieri and Burns, 2003). More recently, ADP-ribosylation has been shown not to be unique to bacterial toxins, but to also occur within mammalian cells (Okazaki and Moss, 1996).

ADP-ribosylation is a posttranslational modification that, analogous to phosphorylation, alters the functional capabilities of target proteins. Four amino acids have been identified as ADP-ribose acceptors for bacterial toxins: arginine, cysteine, asparagine, and a posttranslationally modified histidine residue called diphthamide (Barbieri and Burns, 2003). Some toxins, such as diphtheria toxin, are absolutely specific for their ADP-ribose acceptor (elongation factor-2). Other toxins, such as cholera toxin, may ADP-ribosylate multiple substrates, although modification of one of these, the α-subunit of the heterotrimeric GTP-binding protein, Gs, is the most important for causing the cellular effects leading to the disease cholera. Overall, the molecular basis for ADP-ribose acceptor discrimination remains poorly understood.

ADP-ribosylating AB toxins that kill eukaryotic cells include diphtheria toxin and *P. aeruginosa* exotoxin A. The A fragments of both toxins ADP-ribosylate the unique diphthamide residue of elongation factor-2 to inhibit protein synthesis and trigger cell death, despite a lack of overall sequence homology and overall structural similarities between the two toxins. The consequence of diphtheria toxin action is directly responsible for most of the pathology associated with the disease diphtheria. Other ADP-ribosylating AB toxins modulate eukaryotic cell physiology rather than eliciting a lethal effect. Several of these toxins modify GTP-binding proteins, which are essential in G-protein-coupled signal transduction. Well-characterized examples include cholera toxin, heat-labile toxin, and pertussis toxin, which all ADP-ribosylate the α-subunit of G proteins, leading to increases in cyclic adenosine monophosphate (cAMP), and multiple effects on host cell physiology.

Some type III effectors that are directly injected into the cytosol of eukaryotic cells also exhibit ADP-ribosylating activities. Interestingly, one of these, *P. aeruginosa* ExoS, is a bifunctional cytotoxin, with two independent enzymatic activities: a carboxyl-terminal ADP-ribosyltransferase activity, and an amino-terminal Rho GTPase-activating protein (GAP) (Barbieri, 2000). ExoS

ADP-ribosylates members of the Ras family of small GTP-binding proteins and leads to uncoupling of Ras-mediated signal transduction (Barbieri, 2000). *C. botulinum* C3 toxin catalyses the ADP-ribosylation of Rho proteins involved in regulating the cellular cytoskeleton, which results in functional inactivation of Rho GTPases and the subsequent disassembly of the actin cytoskeleton (Just et al., 2001). These examples illustrate the central importance of GTP-binding proteins as targets of intracellular-acting bacterial toxins.

Intracellular-acting Toxins that Glycosylate Their Targets. Members of another group of toxins catalyze the mono-*O*-glucosylation or mono-*O*-*N*-acetylglucosaminylation of their eukaryotic target proteins, where the "mono-*O*" refers to modification of the hydroxyl–side chain oxygen atom from an acceptor threonine residue (Aktories, 2003). The sugar moieties transferred during these reactions are derived from the substrates UDP-glucose or *N*-acetylglucosamine (in the case of *C. novyi* α-toxin), which are found at relatively high concentrations within eukaryotic cells.

Members of the Ras GTPases, a family of "molecular switches" that regulates various signaling processes within eukaryotic cells, are glycosylated by several bacterial toxins. These small GTPases cycle through "on and off" states, depending on whether GTP or GDP is bound, respectively. In the GTP-bound form, the Ras GTPases interact with numerous cellular effectors, including serine/threonine kinases, lipid kinases, phospholipase D, and several adaptor proteins. When members of the Ras GTPases are glucosylated, the functional consequence is generally to block the ability of these proteins to interact with their respective downstream effectors, which, in turn, inhibits the downstream signaling mediated by these proteins. Thus, various cellular functions, such as regulation of the actin cytoskeleton, are inappropriately altered by toxin action (Aktories, 2003; Juris et al., 2000).

The glucosylating toxins are often referred to as the large clostridial cytotoxins, because of their very large sizes (single polypeptides of approximately 300 kDa). The glucosyltransferase activities are localized to specific domains, consistent with the molecular structure of most AB intracellular-acting toxins. Two of the best-studied large clostridial toxins thus far are Toxin A and Toxin B from *C. difficile*, a pathogen that resides in the human large intestine, and is associated with antibiotic-associated diarrhea, and pseudomembranous colitis, a severe consequence of certain therapeutic antibiotic regimens. Toxin A and toxin B monoglucosylate the Rho GTPase subfamily associated with cytoskeletal regulation, which are Rho, Rac, and Cdc42 (Juris et al., 2000). The *C. sordelli* lethal toxin modifies a second group of small GTPases, Ras, Rap, and Ral, which are associated with the regulation of different cellular functions than the Rho subfamily proteins, although significant cross talk with the Rho-mediated signaling pathways does exist.

Intracellular-acting Toxins that Deamidate Proteins. While members of the large clostridial toxins inhibit the Rho and/or Ras GTPases, other intracellular-acting toxins act instead to activate these same eukaryotic effectors

(Aktories, 2003). These toxins are the cytotoxic necrotizing factors (CNFs) made by various pathogenic *E. coli* strains, and the dermonecrotizing toxins (DNTs) generated from pathogenic *Bordetella* spp., including *B. bronchiseptica*, *B. pertussis*, and *B. parapertussis* (Aktories, 2003). The CNFs catalyze the deamidation of a specific glutamine residue within the Rho-active site, which results in the conversion to a glutamic acid. Deamidation blocks conversion of the activated GTP-bound form to the inactivated GDP-bound form so that Rho is retained in the constitutively active state, which has consequences for downstream signaling involved in regulating the cellular cytoskeleton. In contrast to the CNFs, the DNTs appear to primarily catalyze transglutaminase reactions in which the NH_3 moiety of the targeted glutamine side chains are "swapped" with larger primary amines derived from cellular substrates present in high amounts, such as the amino acid lysine, or the polyamines putrescine, spermine, or spermidine (Aktories, 2003). The larger side-chain moieties block the conversion of the GTP-bound to GDP-bound forms, thereby "locking" the Rho GTPases into the activated state (Aktories, 2003).

Intracellular-acting Toxins that Deadenylate Ribosomal RNA. Several bacterial toxins share an enzymatic activity with plant toxins, which is *N*-glycosidation of a specific ribonucleotide in the 28S RNA of host ribosomes (Melton-Celsa and O'Brien, 2003). The cleavage of a conserved adenine residue (deadenylation) from the well-conserved aminoacyl-tRNA-accepting loop of rRNA results in the inability of the elongation factors to appropriately interact with ribosomes during protein synthesis. This single deadenylation reaction effectively halts protein synthesis in its tracks, resulting in subsequent death of the intoxicated cells. This group of toxins are collectively called ribosome-inactivating proteins (RIPs). Although RIPs share very little sequence homology, their active sites seem to be conserved. A glutamic acid is required for the toxin's enzymatic activity. Several other residues such as arginine, tyrosine, and tryptophan, are also important for the toxins to exert full enzymatic activity. Some RIPs may additionally require "cofactors" such as ATP or ribosomal proteins, and the activity of other RIPs may be enhanced by tRNA.

Bacterial-derived RIPs include Shiga toxin (Stx) produced by *S. dysenteriae*, the causative agent of dysentery, and Shiga-like toxin 1 (Stx1) and Shiga-like toxin 2 (Stx2) produced by *E. coli* (O'Loughlin and Robins-Browne, 2001). These Stx proteins are classic AB toxins with the A fragments responsible for the *N*-glycosidase activities, while the B fragments form pentamers that facilitate the binding of these toxins to the specific receptor globotriaosylceramide (Gb3). Once inside sensitive cells, the toxins are transported via retrograde trafficking into the cytosol to deadenylate ribosomes. Although there is no evidence showing that these toxins act directly on host cell DNA, intoxicated cells always undergo apoptosis, presumably by mechanisms involving the degradation of nuclear DNA by host cell factors.

Intracellular-acting Toxins that Degrade Host Proteins. One of the most direct strategies for disrupting normal cellular functions is for pathogenic bacteria to generate intracellular-acting toxins with protease activities to cleave multiple host protein substrates. In reality, intracellular-acting toxins with protease activities tend to be relatively specific for their substrates, demonstrating again the capacity of toxins to precisely modulate host cell functions.

Some of the most potent intracellular-acting toxins are the zinc-dependent metalloproteases generated by *C. botulinum* (the botulinum neurotoxins, or BoNTs) and *C. tetani* (tetanus neurotoxin, or TeNT) (Rossetto et al., 2001, 2003). These AB neurotoxins specifically cleave eukaryotic VAMP, SNAP 25, or syntaxin, which, during exocytosis, prevents the appropriate docking of membrane-bound vesicles to the cell membrane, and the subsequent release of the neurotransmitter acetylcholine that interacts with receptors across the synapse. Depending on where the toxins act, the consequence of blocking neurotransmitter release is flaccid (BoNTs) or spastic (TeNT) paralysis.

Another example of an important toxin with protease activity is one of the two anthrax toxin A fragments, called lethal factor (LF). LF is a zinc-binding metalloprotease that cleaves the amino terminus of several MAP kinase kinases (MAPKKs), which are critical players in signal transduction pathways controlling cell proliferation and differentiation (Rossetto et al., 2003). However, some debate exists as to whether MAPKKs are the only substrates of LF and, if so, how the modification of these molecules contributes to the symptoms observed from the action of LF.

A final example of toxins elaborating protease activities are the *Yersinia* virulence factors YopJ and YopT, which are type III effector proteins that are injected directly into the cytosol of host cells. YopJ is a ubiquitin-like cysteine protease that cleaves the carboxyl-terminus of the small ubiquitin-related modifier (SUMO), which is ligated to lysine residues in a variety of target proteins after processing (Johnson, 2004). Ubiquitin modification has been correlated with a number of signaling pathways, cell progression, and DNA repair. Although the exact targets of YopJ have not been identified yet, YopJ may cleave and inhibit a component(s) of the NF κB pathway (Orth, 2002), which is suggested by the capacity of YopJ to inhibit the host immune response and induce apoptosis by blocking multiple signaling pathways. YopT is also a cysteine protease, which has been recently shown to cleave Rho, Rac, and Cdc42 at their carboxyl-termini, releasing them from the membrane to result in the disruption of the cytoskeleton within host cells (Shao et al., 2002).

Intracellular-acting Toxins that Degrade Host Nucleic Acids. Another seemingly direct approach for disrupting normal cell function is the production of intracellular-acting toxins that can degrade host nucleic acids. Members of the cytolethal distending toxins, which are generated by a variety of pathogenic species, have been shown to exhibit DNAse activities in vitro, which is probably related to the capability of these toxins to invoke cell cycle arrest in a number of cell lines (Dreyfus, 2003). Cells intoxicated with the cytolethal

distending toxin become enlarged and distended over the course of 3–4 days because of the G2/M cell cycle arrest, and eventually die. A number of pathogens that colonize diverse sites within the host produce cytolethal distending toxins, including *Campylobacter jejuni*, *E. coli*, *Haemophilus ducreyi*, *Actinobacillus actinomycetemcomitans*, and *Heliobacter hepaticus* (Thelestam and Frisan, 2004).

Intracellular-acting Toxins that Degrade Host Lipids. In addition to degrading proteins and nucleic acids, intracellular-acting toxins have been discovered that also degrade host lipids. The hydrolysis of lipids by lipases is ubiquitous in all organisms, and is important for lipid metabolism, signal transduction, and remodeling host membranes. Thus, the inappropriate, toxin-mediated degradation of lipids can cause profound effects within mammalian cells. The effector toxin ExoU from *P. aeruginosa*, which has long been associated with lung injury, bacterial dissemination, and sepsis in animal model and human infections, was recently found to elaborate a lipase activity (Sato et al., 2003). Using yeast as a model system, ExoU caused an accumulation of free palmitic acid, changes in the phospholipid profiles, and a reduction of radiolabeled neutral lipids. Clues as to the activity of ExoU were derived from (1) the reduction of ExoU cytotoxicity with phospholipase A inhibitors, and (2) sequence homology between ExoU and other phospholipases. ExoU is injected directly into eukaryotic cells via a T3SS and, interestingly, seems to require activation or modification by eukaryotic factors.

8.2.3.2. Intracellular-acting Toxins that Act by Mimicking Host Cell Effectors

Several intracellular-acting toxins closely mimic existing host cell effectors. Most likely, the inappropriate elaboration of these activities trigger the modulatory effects observed within host cells.

Intracellular-acting Toxins that Phosphorylate Host Substrates. Because of the central role of phosphorylation-mediated signaling pathways within mammalian cells, it is not surprising that some pathogens produce toxins that mimic some of the most important enzymes involved in these regulatory cascades, which include the kinases. While bacterial histidine kinases are well known to function in two-component regulation of virulence genes, it had been generally believed that bacteria do not possess serine and theronine kinases, which more closely mimic eukaryotic kinases. However, several virulence factors have been demonstrated to have "eukaryotic-like" kinase activities. The best characterized example is the protein kinase YpkA from *Yersinia*. YpkA is an effector protein that is produced as an inactive autophosphorylating serine/threonine kinase. Upon cellular entry, YpkA binds to actin and is converted into a highly active enzyme. The toxin then induces host cell cytoskeleton rearrangements by causing the depolymerization of actin, thus inhibiting macrophage function and phagocytosis of the bacterium (Cornelis, 2002; Juris et al., 2000). In this example, YpkA seems to

be mimicking the eukaryotic RhoA-binding kinases, such as PKN/PRK1, to interact with RhoA (Dukuzumuremyi et al., 2000). However, the YpkA kinase target(s) and its exact mode of action remain unclear.

Intracellular-acting Toxins that Dephosphorylate Host Substrates. Bacterial proteins with activities mimicking those of eukaryotic phosphatases have also been recently discovered. One of the best-studied examples is the protein tyrosine phosphatase YopH from *Y. pestis*. Both YopH and the kinase YpkA are encoded by genes found on a *Yersinia* pathogenicity island, and are injected into the host cell cytosol by a T3SS. YopH dephosphorylates a variety of focal adhesion proteins to disrupt host cell focal adhesions. Thus, by functioning in concert with other *Yersinia* effector proteins acting on monomeric GTPases of the Rho family to modulate the dynamics of the host cell cytoskeleton, YopH acts to protect *Yersinia* against phagocytosis (Cornelis, 2002).

Another example of a bacterial toxin that dephosphorylates eukaryotic substrates is the type III effector protein SopB from *Salmonella* spp., which exhibits inositol phosphatase activity in vitro. SopB shares sequence homology with eukaryotic cell inositol phosphate 4-phosphatase, and hydrolyzes phosphatidylinositol triphosphate (PIP3) to alter inositol phosphate metabolism, thus activating the calcium-dependent chloride secretion pathways (Norris et al., 1998). SopB also mediates actin cytoskeletal rearrangements and bacterial entry in a Cdc42-dependent manner (Zhou et al., 2001). *Shigella* IpgD is homologous to SopB, and may have a similar role in pathogenesis.

Intracellular-acting Toxins with Adenylate Cyclase Activity. Several intracellular-acting toxins catalyze the excess production of the potent intracellular second messenger, cAMP, resulting in the alteration of major cellular functions. cAMP levels in eukaryotic cells are maintained under tight regulation, because they are involved in many cellular functions such as Ca^{2+} influx, gene expression, and cell-specific processes such as glycogenolysis and lipolysis (Rich and Karpen, 2002). We have previously discussed the *Bordetella* adenylate cyclase toxins (ACTs), which are apparently bifunctional toxins comprising a calmodulin-dependent adenylate cyclase domain fused to a pore-forming hemolysin. Upon entering eukaryotic cells, the catalytic domains of these toxins trigger a large increase in cAMP levels, leading to an inhibition of chemotaxis and depression of mammalian cell functions such as superoxide generation (Confer and Eaton, 1982). Another interesting example of a calmodulin-dependent adenylate cyclase is the *B. anthracis* edema factor (Bhatnagar and Batra, 2001). Recall that edema toxin is generated from the binary association of edema factor with the toxin's B fragment, protective antigen. While the exact role of edema factor is not yet clear, there is some evidence that the toxin might act to modulate the immune response to *B. anthracis* infection.

Intracellular-acting Toxins that Mimic Host GTP-Exchange Factors. As we have already discussed, the Rho GTPases are important targets for a number

of bacterial toxins, primarily because of the important roles of these small GTP-binding proteins in regulating important cellular processes. In addition to those toxins that covalently modify the Rho GTPases, there is another class of toxins that noncovalently modulate the properties of these proteins by mimicking endogenous eukaryotic regulatory factors of Rho GTPases. The actions of these toxins are therefore reversible.

One such class is the GTP exchange factors (GEFs), which facilitate the exchange of GTP for GDP bound to unactivated GTPases. Several type III effectors, including SopE and SopE2 from *Salmonella* spp., have been identified to function as GEFs. Along with the protein tyrosine phosphatase SopB, SopE and SopE2 trigger actin polymerization and membrane ruffling, leading to bacterial invasion. SopE activates Rac and Cdc42, while SopE2 is specific for Cdc42 (Bliska and Viboud, 2003).

Intracellular-acting Toxins that Mimic Host GTPase-activating Proteins. Several other bacterial toxins mimic the eukaryotic GTPase-activating proteins (GAPs). GAPs increase the GTPase activity of the activated form of the small GTP-binding proteins, thus converting them into the unactivated GDP-bound forms. Several type III bacterial effector proteins have GAP activities for Rho GTPases, such as SptP from *S. enterica*, ExoS and ExoT from *P. aeruginosa*, and YopE from *Yersinia* spp. The action of these toxins inhibit actin polymerization in host cells. The bacterial GAPs share a number of common features including a GAP-like arginine finger motif that is essential for GAP activity, as well as a common mechanism for interacting with members of Rho GTPases (Bliska and Viboud, 2003).

8.2.3.3. Intracellular-acting Toxins that Act by Unknown Functions

As intracellular toxins continue to be discovered, there will likely be toxins that modulate host cells by previously undescribed mechanisms. One example is the *H. pylori* vacuolating cytotoxin (VacA), which can elaborate cellular cytotoxicity when expressed directly within the cytosol of target cells. However, unlike the intracellular-acting toxins that we have discussed previously, there has not been an enzymatic activity yet associated with VacA. In fact, there is significant evidence that VacA may elaborate an intracellular channel-forming activity that is directly required for at least some of the cellular phenotypes induced by the toxin. VacA's channel activity is not apparently associated with the toxin's capacity to enter mammalian cells; nor is it likely to facilitate the translocation of the toxin's catalytic domain (if any) into the cytosol, as is the case for AB toxins such as diphtheria, anthrax, and the botulinum neurotoxins. VacA was originally discovered because of the capacity of the toxin to induce degenerative vacuolation of sensitive mammalian cells. Channel blockers partially inhibit the rate of vacuole biogenesis, indicating that cellular vacuolation is dependent on channel formation (Tombola et al., 1999a, b, 2000). In addition to inducing vacuolation, VacA also induces apoptosis in gastric epithelial cells (Cover et al., 2003) by a

mitochondria-dependent pathway, involving the release of cytochrome *c* from mitochondria and activation of caspase 3 (Galmiche et al., 2000). Recent data demonstrated that, similar to cellular vacuolation, VacA-mediated changes in mitochondrial membrane permeability resulting in apoptosis are also dependent on functional VacA channels (Willhite et al., 2003), indicating that toxin channel activity is essential for the toxin to elicit multiple cellular effects. Moreover, VacA enters cells and localizes to mitochondria, suggesting that VacA may form channels directly in the mitochondrial membrane. Interestingly, such an activity is reminiscent of several pro-apoptotic Bcl-2 proteins (Tsujimoto, 2002). VacA is therefore an interesting example of a bacterial toxin whose primary effects within host cells are the direct consequence of toxin channel activity at one or more intracellular organelles, rather than the elaboration of an enzymatic activity.

9. How Do Bacterial Toxins Contribute to the Virulence of Pathogenic Bacteria?

As we have seen, pathogenic bacteria generate bacterial toxins with a diverse array of biochemical activities in order to modulate host cells. Clearly, tremendous progress has been made in understanding the molecular aspects of toxin action and the consequences of intoxication on host cellular function. However, as we have pointed out several times already, assessing the exact roles of some toxins in virulence remains a complicated proposition at best. For example, how can one rationalize any benefits that would come to *C. botulinum* from generating neurotoxins that cause extensive host pathology in the absence of infection? In other cases, toxins have been demonstrated to induce many alterations in target cells. Are all of these observed effects relevant to pathogenesis? Only in very few cases are there sufficient data to provide a reasonable view of how the cellular modulatory activity of a particular toxin might contribute to the virulence of the pathogenic organism. Several examples of how toxin action may contribute to pathogenesis will be discussed. However, the wise reader will keep in mind that these models only represent attempts to rationalize and bring some order to a large number of complex phenomena.

9.1. Toxins Facilitate the Acquisition of Nutrients for Bacterial Colonization

The acquisition of sufficient nutrients within a host is important for the success of microbes during an infection. Some toxins facilitate the release of nutrients from host cells that can be scavenged by pathogens at the site of infection. Access to nutrients is especially critical when the host hordes iron, which is an essential element for the survival of most bacteria and eukaryotic cells. We have already discussed how *C. diphtheriae* regulates the expression of

the diphtheria toxin gene by directly sensing the amount of available iron (Tao et al., 1994). Under conditions of low iron, diphtheria toxin is synthesized and released, resulting in the efficient killing of host cells in the upper respiratory tract. As a consequence of cell killing, nutrients are released and presumably used by *C. diphtheriae*. When iron concentrations reach a sufficient level, the transcription of diphtheria toxin is halted because the toxin is no longer needed. Some pore-forming toxins may also allow nutrients to leak out of host cells to be used by the bacterium. Examples of pore-forming toxins proposed to contribute to bacterial survivial in this manner include aerolysin from *A. hydrophila,* and α-hemolysin from *S. aureus* (van der Goot, 2003).

9.2. Toxins that Facilitate Bacterial Infection and Dissemination by Remodeling of Host Colonization Niches

The epithelial membrane provides a barrier that is normally impassable to pathogenic microbes, and thus protects the underlying tissues and bloodstream. However, some pathogens apparently use toxins as a means to remodel the epithelial environment. "Remodeling" here is meant to convey that toxins actually alter the physiological, biochemical, and structural properties of the epithelial membrane.

Several toxins act to loosen the tight junctions that serve as the molecular glue between neighboring cells. Recall that the enterotoxin BFT from *Bacteroides* spp. is a protease that ultimately cleaves the extracellular domain of E-cadherin (Rappuoli and Pizza, 2000). A second example is the *C. perfringens* enterotoxin, which interacts with claudins and occludins at tight junctions, ultimately resulting in the "loosening" of the membrane (McClane, 2001). Conceivably, the consequences of altering tight junctions might be to disrupt the membrane barrier separating the intestinal lumen from the underlying tissues, and thus promote dissemination of microbes within the host.

Toxins may act to remodel host niches by several other mechanisms. Recall that VacA from *H. pylori* enters mammalian cells and localizes to the mitochondria and causes changes at this organelle consistent with the induction of apoptosis (Galmiche et al., 2000; Willhite et al., 2003). VacA-induced apoptosis in acid-secreting parietal cells has been suggested to be important for *H. pylori* colonization within the stomach (Boquet et al., 2003; Neu et al., 2002; Salama et al., 2001). Because parietal cells are abundant in mitochondria, which produce ATP required for the pumping of protons within the gastric lumen, these cells may be especially susceptible to the modulating activity of VacA. In fact, acid secretion from isolated parietal cells is reduced when exposed to VacA-expressing *H. pylori* strains (Kobayashi et al., 1996). Thus, VacA may facilitate *H. pylori* colonization within the stomach by acting to prevent a drop in pH, which is important because, although *H. pylori* are successful gastric pathogens, the organisms are not acidophilic.

9.3. Toxins that Facilitate Colonization and Persistence Through Modulation of the Immune Response

An emerging paradigm is that bacterial toxins can modulate host responses to the presence of the pathogenic microbes by acting directly upon immune cells. For example, it was recently demonstrated that *B. anthracis* lethal toxin targets MAPKKs in dendritic cells (Agrawal et al., 2003), which are then no longer able to assist in the adaptive immune responses during *B. anthracis* infections. Another example may be the *H. pylori* vacuolating cytotoxin, which was also recently demonstrated to block proliferation of T cells by inducing a G1/S cell cycle arrest (Gebert et al., 2003). This form of immune suppression would likely contribute to the chronic nature of *H. pylori* infections within the human stomach. Additional examples are the aerolysins produced by a variety of *Aeromonas* spp., which are examples of pore-forming toxins that are capable of causing death of various types of host cells, including immune cells, by several mechanisms, including lysis due to the development of pore-induced osmotic imbalances (Fivaz et al., 2001). Finally, the degradation of immune cell-surface molecules by some membrane-disrupting toxins may be a mechanism utilized by pathogenic bacteria to avoid host defenses. For example, some phospholipase C (PLCs) exhibit cytolytic activity toward host phagocytic cells in vitro.

9.4. Toxins that Facilitate Intracellular Lifestyles

Some toxins facilitate the invasion or escape of pathogenic bacteria from eukaryotic cells within the host. There are multiple examples of type III effectors that act to manipulate host cells during the different stages of a pathogen's intracellular adventure. Effectors injected into host cells alter the host cytoskeleton to facilitate the uptake process, as has been shown for a number of pathogens, including *Salmonella* spp. (SopB, SopE, and SopE2) and *Shigella* (VirA) (Yoshida and Sasakawa, 2003; Zhou and Galan, 2001). Toxins also contribute to the escape of microbes from the inside of host cells by a variety of mechanisms. For example, *Listeria monocytogenes*, which is taken up into cells initially by an endocytic-like process, must escape the membrane-bound vesicles that transport the bacteria into the cells to be able to ultimately disseminate into neighboring cells (Cossart, 2002). Escape into the cytosol requires production of listeriolysin O, which forms a pore in the membrane of the phagosome, allowing the bacteria to escape (Portnoy et al., 2002; van der Goot, 2003). A second example is the pore-forming toxin IcmS produced by *Legionella pneumophila*, which is phagocytosed into macrophages. Once the bacterium has proliferated within the cytosol of the macrophage, IcmS punches a hole in the macrophage plasma membrane from the cytoplasmic side to facilitate the release of bacteria into the extracellular environment (van der Goot, 2003). But there are indirect mechanisms as well, such as the induction of macrophage apoptosis by the toxin effector SipB from *Salmonella*. While residing within the phagosome, the bacteria inject

molecules of SipB into the cytosol, which then induce cell death by an unusual mechanism (Santos et al., 2001). Presumably, death of the macrophage allows *Salmonella* to escape the membrane-bound vesicle of the macrophage into the extracellular environment at the appropriate time and place.

10. The Benefical Manipulation of Toxins as Molecular Tools in Medicine and Basic Science

Understanding how toxins work has required the skills of scientists from numerous fields, including molecular genetics, biochemistry, biophysics, structural biology, and cell biology. However, the payoff has been considerable, as the capacity of toxins to modulate eukaryotic cells has been harvested for applications benefiting several disciplines, including cellular microbiology, pharmacology, signal transduction, vaccinology, and immunology. Several of these applications are discussed below.

10.1. The Use of Toxins as Vaccine Adjuvants

Modified forms of cholera toxin and the *E. coli* heat-labile toxin are frequently used as vaccine adjuvants. Adjuvants are components added to vaccines in order to enhance the immune response to the primary antigens. The use of cholera toxin or heat-labile toxin as vaccine components might initially strike the reader as a pretty questionable practice. However, the unraveling of the structure–function relationships underlying the mechanisms of toxin action has promoted "rational detoxification" approaches to eliminate potential harmful effects of cellular intoxication within the host, while retaining the ability of the altered toxins to stimulate strong immune responses as adjuvants. Recall that cholera toxin and heat-labile toxin are AB toxins. Separation of the A and B fragments results in two nontoxic moieties, and, in fact, the B fragments alone are effective adjuvants (Wang et al., 2001). However, a more sophisticated strategy has been to detoxify the A fragments by generating mutations at specific amino acids that are important for enzymatic activity, resulting in altered forms of the toxins that are no longer capable of modifying their intracellular targets. Such strategies are important because the detoxified whole toxin (i.e., both A and B fragments present) has been revealed to be a better adjuvant than the B fragment alone (Del Giudice and Rappuoli, 1999). Additional strategies that may be useful include altering the toxin to prevent the processing and separation of the toxin's A and B fragments, rendering the toxins incapable of transporting the catalytic A fragments into the cytosol of target cells (Del Giudice and Rappuoli, 1999).

10.2. The Use of Toxins as Magic Bullets

The potency of toxins such as diphtheria toxin and *Pseudomonas* exotoxin A in killing target cells has prompted their use as "magic bullets" for cancer immunotherapies (FitzGerald et al., 2004). The magic bullet strategy has

generally involved coupling the enzymatic A fragments of these toxins to the Fab portion of antibodies selectively targeting antigens found only on the surface of tumor cells. The resulting "immunotoxins" should discriminate for cancer cells, which would be selectively killed by the A fragments, while leaving "normal" cells unharmed. This approach has met with some success, although a major obstacle seems to be the inefficient translocation of the toxic cargo into the cytosol of target cells.

10.3. The Use of Toxins as Delivery Vectors

The remarkable capability of bacterial AB toxins to mediate the cytosolic translocation of catalytic fragments has prompted the manipulation and use of these toxins as delivery vectors to efficiently transport heterologous proteins and peptides into mammalian cells. The overall strategy has generally been to genetically or chemically replace toxin A fragments with alternative molecular cargo that can be delivered safely and efficiently into target cells by fully functional B fragments.

The anthrax toxin has been engineered into an epitope delivery system (Goletz et al., 1997). Recall that the anthrax toxin is a binary toxin, which requires that the A and B fragments interact at the plasma membrane to successfully intoxicate target cells. Structure–function analysis has revealed that the LF amino-terminal domain mediates the association between the carboxyl-terminal metalloprotease domain and the toxin's B fragment (protective antigen) (Bhatnagar and Batra, 2001). When T-cytotoxic lymphocyte-stimulating epitopes are substituted at the LF's carboxyl terminus, the protective antigen successfully delivers the epitopes into cells for processing and MHC class I presentation. Studies with epitopes derived from various pathogens demonstrated that PA-mediated delivery stimulates a T cell response with the capacity to be protective (Ballard et al., 1996, 1998).

A second example that we have already discussed several times is the *B. pertussis* adenylate cyclase toxin, which directly translocates the toxin's adenylate cyclase catalytic domain into the cell cytosol from the plasma membrane. The adenylate cyclase toxin receptor, CD11b/CD18, is present on many cell types associated with the immune response, including macrophages and dendritic cells. The transport machinery of the adenylate cyclase toxin has been engineered as a nonreplicative vector to deliver exogenous epitopes from pathogens or tumors into antigen presenting cells (El Azami El Idrissi et al., 2002).

A final example is the use of the large pore-forming toxins to build "doors" into cells. Recall that the large pore-forming toxin, streptolysin O, has been proposed to function as a gateway for the delivery of other streptococcal virulence factors into target cells (Walev et al., 2001). Several large pore-forming toxins have actually been shown to be useful for opening up temporary doors within the plasma membrane so that various large molecules (such as

proteins) can be introduced into cells (Gauthier and Finlay, 2001). These types of protein transfection systems using large pore-forming toxins are becoming increasingly useful for applications in cell biology.

10.4. Toxins as Molecular Reagents in Cell Biology and Pharmacology

Cell biology and pharmacology have been rich beneficiaries of toxin research. The ability to modulate signal transduction (cholera toxin, pertussis toxin, etc.), vesicle trafficking (botulinum toxin, tetanus toxin, etc.), the cytoskeleton (*C. difficile* ToxB, cytotoxic necrotizing factor, etc.), as well as other important cellular processes at precisely defined steps, have made toxins invaluable reagents for scientists within these fields. Toxins have been especially useful for delineating the roles of GTP-binding proteins in numerous processes within eukaryotic cells, because of the ability to turn on and off these key regulatory switches in a very precise manner.

11. Novel Countermeasures Against the Insidious Uses of Toxins and Toxin-Producing Microbes for Biological Warfare

The potency of some bacterial toxins renders them innately harmful to humans. Unfortunately, recorded history indicates that the use of toxins and toxin-producing bacteria as disease-causing agents for biological warfare is not a new idea. The Centers for Disease Control have classified *B. anthracis*, *Y. pestis*, and the botulinum neurotoxins as high-risk agents and threats to both national security and public health. In the context of bioweapons, *B. anthracis* and *Y. pestis* function as delivery vehicles for toxins (see Chapter 15).

The potential for the deliberate and destructive use of toxins and the toxin-producing microbes was dramatically illustrated in the months following the horrific events of September 11, 2001. These incidents raised national awareness to the need for effective countermeasures to prevent and treat diseases associated with high-risk biological agents and their products. Historically, strategies for controlling bacterial infections within a host have centered on antibiotics that prevent microbial growth. In contrast, relatively little effort has been expended on neutralizing the effects of toxins released from organisms, although in some cases, these virulence factors may be responsible for most of the symptoms associated with the disease.

In the case of *B. anthracis*, mounting evidence indicates that blocking the effects of the anthrax toxins, especially the lethal toxin, could provide significant protection to an infected individual. In fact, new strategies to neutralize anthrax toxin have demonstrated significant promise. One strategy has been to identify dominant-negative mutants of the anthrax protective antigen

(Orlandi), the toxin's B fragment, as potent inhibitors, (Sellman et al., 2000). These inactive forms of PA retain the ability to interact with wild-type toxin monomers, but in doing so, block cellular intoxication by "poisoning" the capacity of toxin complexes to translocate toxin A fragments into cells. A second strategy has been to create synthetic polymers designed to block the action of the toxin (Mourez et al., 2001). These synthetic polymers target the receptor-binding site on PA, and effectively interfere with the association of anthrax toxin with target cells. A third strategy has been to use fragments of the anthrax toxin receptor as drugs to competitively inhibit the binding of the toxin to the plasma membrane of target cells (Chaudry et al., 2002). All three of these clever strategies are clear examples of how investigating the structure–function relationships and cellular intoxication mechanisms underlying toxin action can directly lead to the development of effective countermeasures against the use of toxins for premeditated and destructive purposes.

12. Conclusions

Toxins are important virulence factors in bacterial pathogenesis. While some toxins ultimately kill their target cells, many others act instead to modulate important physiological or structural properties of eukaryotic cells without inducing cell death. Although modulating cell function may not directly kill intoxicated cells, substantial consequences to the host may still occur, underscoring the importance of considering the "global" effects of toxin action within the host.

Subsequent to export from bacterial cells, toxins interact with sensitive host cells and exert biochemical activities that cause alterations in important cellular properties, which, in turn, can result in several changes within the host. Although there are many molecular targets for toxins, these virulence factors can be classified by the overall eukaryotic cell systems, or properties, that are most often affected. Common targets include the plasma membrane, cytoskeleton, protein synthesis machinery, vesicle/membrane trafficking, and cellular signaling and regulatory pathways. The specificity with which many toxins interact with host cell components, and the manner in which some toxins mimic "eukaryotic-like" functions, makes it tempting to speculate that some toxins may have derived from eukaryotic origins. Alternatively, other toxins may have descended from factors that evolved originally to kill other bacterial species.

Although a clear understanding of the role of toxins in pathogenesis can sometimes be difficult, elucidation of the environmental cues that stimulate toxin production can provide clues as to when and where within a host the toxin is needed. In addition, the mode by which a bacterial cell exports a toxin can also hint at where a toxin may act. Toxin effectors secreted by type III systems are "eukaryotic" in nature, and are injected directly into target cells, allowing bacteria to efficiently modulate host cells at the exact site of

colonization. In contrast, toxins exported into the extracellular host environment can exert a broader "sphere of influence" by intoxicating many cells that may not directly be located at the site of infection. The extracellular-acting toxins exert their effects at the plasma membrane or extracellular matrix, while AB intracellular-acting toxins must first enter cells and translocate into the target cell cytosol to elaborate their activities. The cellular intoxication mechanisms of AB intracellular-acting toxins are complex, and involve the use of host cell receptors, uptake and trafficking pathways, and properties of either endosomal vesicles or the endoplasmic reticulum for the translocation of a catalytic fragment into the cytosol of target cells.

Extracellular-acting toxins remodel the plasma membrane of target cells by forming transmembrane pores or, alternatively, degrading cell-surface components. Both the injected effectors of T3SSs and T4SSs and the AB intracellular-acting toxins modulate host cells by elaborating specific enzymatic activities. Some of the more prevalent enzymatic activities include the ADP-ribosylation of target molecules, the protease-mediated breakdown of cellular protein components, and the overproduction of the important secondary messenger, cAMP. While the importance of many toxins is evident, their exact contributions to pathogenesis and the virulence strategies of the organisms that generate toxins is considerably murkier. Potential roles for bacterial toxins may include acquisition of nutrients for the bacterium, remodeling of the host environment, suppression of the host's immune response, and facilitation of intracellular lifestyles.

The remarkable intoxication properties of bacterial toxins have been harnessed in some cases for beneficial and useful applications. A detailed molecular understanding of the cellular intoxication mechanisms of toxins will facilitate the development of effective countermeasures to limit the successful use of toxins and toxin-producing microbes as bioweapons.

Questions to Consider

1. What is the rationale for speculating that some intracellular-acting toxins may derive from eukaryotic origins?

Perhaps the most compelling argument is the exquisite specificity that some toxins demonstrate for both the receptors they bind to on the cell surface and the intracellular targets they modify. Because many toxins bind to plasma membrane receptors as the first step of cellular intoxication, the derivation of receptor selectivities and affinities suggests that the receptor-binding domains of these toxins may have descended from cognate eukaryotic ligands that normally bind these same receptors. Moreover, intracellular-acting toxins sometimes demonstrate high selectivity for eukaryotic target molecules they presumably never encounter outside the host. These observations suggest that some toxins may have derived substrate selectivity by "hijacking" functional domains originating from ancestral or existing eukaryotic molecules. This scenario is especially attractive for toxin effectors injected into

mammalian cells by T3SSs, some of which exhibit eukaryotic-like enzymatic activities that are not normally found in prokaryotic cells, such as tyrosine phosphatase activity, or GEF/GAP activities.

2. Why is it important that bacterial pathogens regulate toxin productions?

The inappropriate expression of toxins at the wrong time or place is a waste of precious resources and energy for bacteria, especially within environments where these commodities may be scarce. Moreover, the inappropriate killing or modulation of host cells may "alert" the immune response in a manner that is not beneficial to the pathogen, for example, by inducing a protective response that is sufficient to clear the infection before the population of pathogenic bacteria has reached sufficient numbers to withstand such a response.

3. Why are intracellular-acting toxins not all secreted directly into the cytosol of host cells?

Bacterial toxins that are secreted directly into target cells can modulate only those cells with which they have formed intimate relationships. In contrast, intracellular-acting toxins that are exported into the extracellular environment would be expected to exert a wider "sphere" of influence by intoxicating cells both at the site of infection and distant to the site of infection. This may be especially beneficial for pathogens that colonize at a specific host site for modulating the properties of cells involved in the immune response.

4. Why are small GTP-binding proteins such common targets for intracellular-acting AB toxins?

Small GTP-binding proteins are key molecular switches within eukaryotic cells for many important processes, and represent important checkpoints between the plasma membrane and nucleus. For example, Rho, Rac, and Cdc42 regulate changes in the actin cytoskeleton, and these proteins are targets for several toxins. The ability of toxins to either inhibit or stimulate the small GTPases allows pathogenic microbes to modulate many important cellular processes in a very specific manner.

5. Describe two overall therapeutic strategies to block the action of intracellular-acting toxins that must assemble into higher-ordered oligomer during cellular intoxication.

(1) Dominant-negative mutants. The goal would be to identify mutant forms of the toxin that can no longer induce cellular modulatory effects, yet retain the capability of interacting with wild-type toxin monomers. A defining property of a dominant-negative inhibitor here would be that a single inactive monomer would be sufficient to "poison" the entire higher-ordered assembled complex otherwise comprising of wild-type monomers.
(2) Blocking the initial interactions of a toxin with sensitive host cells. The three-dimensional structures of toxin receptor-binding domains would

facilitate the "rational design" of inhibitors to target the receptor-binding determinants. Alternatively, one could use combinational chemistry techniques to identify inhibitors even in the absence of structural data. Finally, therapeutics toxins derived from receptor exodomains could be used as competitive inhibitors to block the binding of toxins to sensitive cells.

6. Why might some toxin-derived therapeutics and delivery vectors be efficacious for only a single use within the host?

The answer here depends on whether the immune system recognizes toxin-based therapeutics as foreign antigens and can then mount a neutralizing immune response. Even if this is the case, an effective primary adaptive immune response takes a week or more to develop upon encountering the reagent for the first time, thus allowing a reasonable amount of time for the reagent to be effective. In contrast, the host would be predicted to mount a rapid and effective secondary immune response upon encountering the toxin-based reagent for a second time. For the same reason, if an individual has already been immunized against the toxin or toxin-producing agent from which the agent was derived, this may also prevent the use of the same toxin-based reagents. To prevent immune recognition and promote immune tolerance, the surface of toxins could conceivably be engineered to disguise immunoreactive epitopes.

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Chapter 11

Quorum Sensing: Coordinating Group Behavior Through Intercellular Signals

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Historical Landmarks

1965 "Competence factor" (Com system) of *Streptococcus pneumoniae* described (Tomasz, 1965).

1970 "Autoinducer" control of luminescence in *Vibrio fischeri* described (Nealson et al., 1970).

1981 The structure of *V. fischeri* autoinducer identified as *N*-3-oxo-hexanoyl-L-homoserine lactone (Eberhard et al., 1981).

1983 Cloning of the quorum sensing *lux* genes from *V. fischeri* (Engebrecht et al., 1983).

1994 The term "quorum sensing" introduced to describe LuxR-LuxI cell density dependent gene regulation (Fuqua et al., 1994).

1995 Sequence of the competence-stimulating peptide (CSP) determined for *S. pneumoniae* (Håvarstein et al., 1995).

2002 Structure of AI-2 (Autoinducer-2) determined for *V. harveyi* (Chen et al., 2002).

1. Introduction

1.1. Quorum Sensing (QS) Is a form of Cell-to-cell Communication that Coordinates Gene Expression Among Groups of Cells

The term "quorum sensing" is commonly used to describe intercellular signaling because activity has been shown in liquid batch culture to be dependent upon the bacterial cell density, and a minimum population, or quorum, is required to initiate signaling (Fuqua and Greenberg, 2002; Fuqua et al., 2001; Miller and Bassler, 2001; Taga and Bassler, 2003). Therefore, bacteria are capable of monitoring their own population density and use this form of communication to coordinate expression of particular genes. Many different genes and processes are regulated by quorum sensing for different bacterial species. Additionally, many quorum sensing–regulated genes encode secreted factors. Presumably, these factors would be ineffective if secreted by a single bacterium.

1.2. QS Involves Production of an Extracellular Signal that Affects a Concentration-dependent Response

In general, production of signal molecules occurs at some basal level. A signal response is initiated when signal concentration reaches a critical threshold. In liquid batch culture, as bacteria grow and the population increases, the concentration of signal increases. Quorum sensing signal generation is often positively autoregulated. This is thought to ensure that all bacteria in the local population are rapidly induced following induction of a subpopulation (Fuqua et al., 1994, 2001; Miller and Bassler, 2001).

2. Acyl-Homoserine Lactone (AHL) QS in Gram-negative Bacterial Species

2.1. A Common Type of Gram-negative QS Involves AHL Signal Molecules

The acyl-homoserine lactone (AHL) structure consists of a fatty acid chain with an amide bond linkage to a lactonized homoserine (Fuqua and Greenberg, 1998, 2002; Fuqua et al., 2001; Whitehead et al., 2001). AHL signaling appears to be highly conserved among the Proteobacteria—greater than 50 species have been identified to produce AHLs (Table 1) (Fuqua and Greenberg, 2002). AHL molecules are described as amphipathic, since the homoserine lactone ring is hydrophilic and the acyl side chain is hydrophobic. This amphipathicity appears to facilitate free diffusion of AHLs within aqueous environments, as well as across the phospholipid bilayer of

cell membranes. The AHL, *N*-3-oxo-hexanoyl-L-homoserine lactone, of *Vibrio fischeri* was the first AHL quorum-sensing signal whose structure was determined (Eberhard et al., 1981).

There is great variability between the AHL structures produced by different species and even among some AHLs produced by the same species. While the homoserine lactone ring is always conserved, acyl side chains species range from 4 to 14 moiety carbons in length, typically containing an even number of carbons (Fuqua et al., 2001). This side chain length has been shown to be important for AHL signal recognition, in particular for bacteria that use multiple AHL systems, such as *Pseudomonas aeruginosa* (Fuqua et al., 2001). Besides length, acyl side chains also display variability in structure (Fuqua et al., 2001). Acyl side chains have been shown to exhibit flexibility at the three-carbon position, which can contain a hydroxyl or ketone group. The acyl side chain can also vary in the degree of saturation.

2.2. LuxI Family Members Are AHL Synthases

The LuxI protein of *V. fischeri* and homologous proteins are responsible for production of AHL signal molecules. Genes encoding LuxI homologs have been identified in over 40 species (Geer et al., 2002). There are ten highly conserved residues toward the amino terminus of LuxI family proteins (e.g., R25, F29, W35, E44, D46, D49, R70, F84, E101, and R104 of *V. fischeri*; (see Figure 1 (Fuqua and Greenberg, 2002)), and it has been suggested that the divergence in the carboxy terminal region may be related to the recognition of different fatty acid substrates required for the synthesis of the different acyl side chain length AHLs (Hanzelka et al., 1997). The two substrates required for AHL synthesis are *S*-adenosylmethionine (SAM) and an acylated acyl carrier protein (acyl-ACP) (Hanzelka and Greenberg, 1996; Moré et al., 1996; Schaefer et al., 1996). SAM is a common cellular metabolite that donates an amino group and the homoserine lactone ring to AHL synthesis. The enzymatic reaction is thought to initiate through SAM binding to LuxI, which then binds acyl–ACP (Fuqua et al., 2001). The acyl group of acyl-ACP is transferred to the SAM/LuxI complex, releasing free ACP. After lactonization of this secondary Acyl/SAM/LuxI complex, the newly synthesized AHL molecule is released leaving a 5′-methylthioadenosine (MTA)/LuxI complex. LuxI family proteins then release MTA as a reaction byproduct (Figure 1) (Fuqua et al., 2001). Alternatively, analysis of the crystal structure for EsaI, a LuxI family member of *Pantoea stewartii*, suggests that EsaI binds acyl-ACP first and binds SAM second. This proposed mechanism is based upon structural similarity of EsaI to *N*-acetyltransferases that bind phosphopantetheine. Phosphopantetheine is a component of coenzyme-A, the prosthetic group by which acyl side chains are covalently linked to ACP (Watson et al., 2002)).

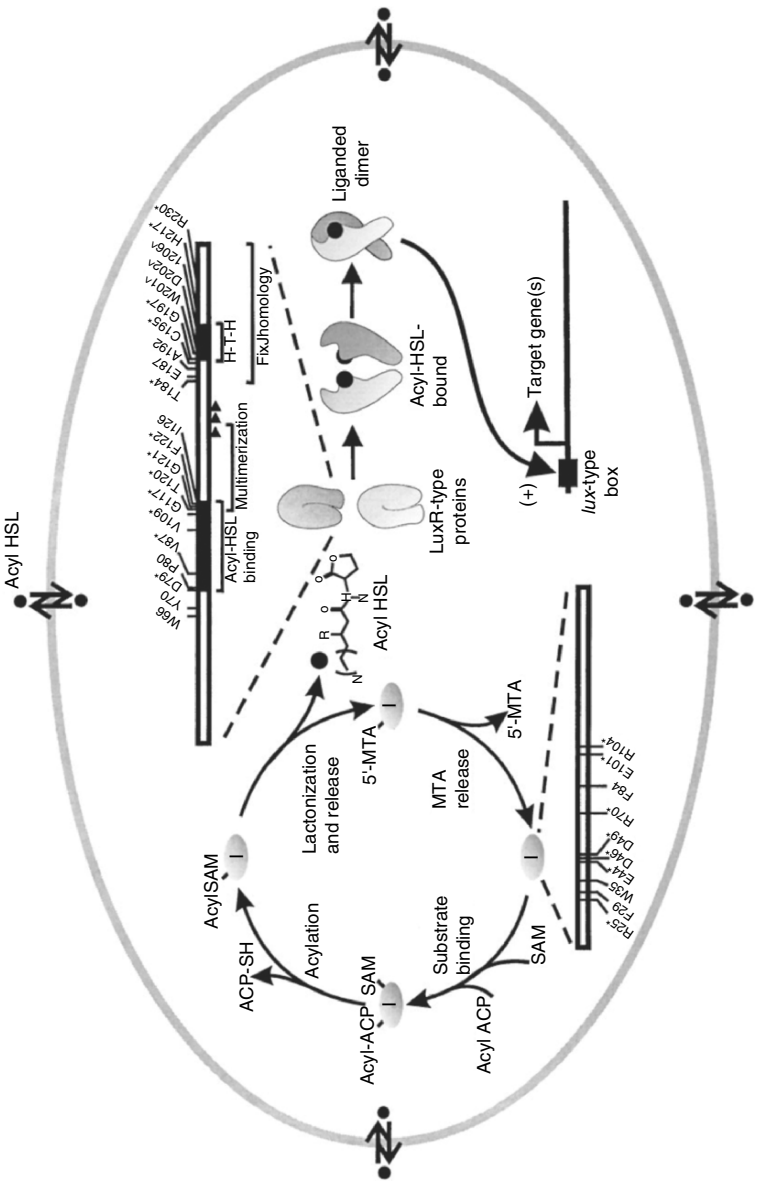


FIGURE 1. Quorum sensing in Gram-negative bacteria (From Fuqua et al., 2001. Reprinted, with permission from Annual Reviews; ©2001; www.annualreviews.org)

Recent evidence has pointed to the role of efflux proteins in the secretion of AHL signal from the cell (Evans et al., 1998; Pearson et al., 1999). Active efflux may be of greater importance for AHL molecules with longer, more hydrophobic, side chains (Fuqua and Greenberg, 2002). It is also important to note that AHL synthesis can be driven by proteins not homologous to LuxI. AinS, LuxM, and VanM of *V. fischeri*, *V. harveyi*, and *V. anguillarum*, respectively, constitute a separate family of AHL synthases (Bassler et al., 1994; Gilson et al., 1995; Hanzelka et al., 1999; Milton et al., 2001).

2.3. LuxR Family Members are AHL Signal Receptors

Perception of the AHL signal occurs through a DNA-binding receptor protein. The LuxR protein of *V. fischeri* (and homologous proteins in other bacteria) binds to specific DNA sequences proximal to quorum-sensing regulated promoters (Hanzelka and Greenberg, 1995; Slock et al., 1990). LuxR family members also bind AHLs and, depending upon the system, can induce activation of a promoter or stimulate the LuxR homolog to release DNA, relieving repression of the regulated promoter.

The LuxR protein of *V. fischeri* consists of 250 amino acids, and two highly conserved functional regions have been identified. These critical regions act to (1) bind AHL signal (residues 66-138 of LuxR); and (2) bind to DNA (residues 183-229 of LuxR) (Fuqua et al., 2001). Amino acid sequence homology of these two regions among LuxR-type proteins is significantly greater than the overall sequence similarity of 18–23% (Fuqua et al., 2001). LuxR family proteins are actually part of a larger group (FixJ-NarL family) that, in general, are two-component-type response regulators (Fuqua et al., 2001).

LuxR family proteins have four distinct functions that have been identified: (1) recognition and binding of AHL signal; (2) conformational and/or multimerizational changes resulting from AHL binding; (3) binding or release of specific regulatory DNA sequences of target promoter regions; and (4) activation or repression of target gene transcription (Fuqua et al., 2001). Binding of AHL signal occurs toward the amino-terminal end of LuxR family proteins (Fuqua et al., 2001). DNA binding and transcriptional activitation utilize carboxy-terminal residues (Fuqua et al., 2001). This region shows the highest homology to other FixJ proteins (mentioned above) and contains a helix-turn-helix motif. Upon binding AHL, some LuxR family proteins form multimers (Fuqua et al., 2001). This has been shown for TraR of the plant pathogen *Agrobacterium tumefaciens* that forms a dimer upon AHL binding with each monomer consisting of two symmetrical folded domains that are linked at a 90° angle (such that the dimer is asymmetrical). TraR is the only crystal structure determined to date for a LuxR family member. The AHL-binding domain of TraR (at the amino terminus) has an $\alpha/\beta/\alpha$ secondary structure, while the carboxy terminus that binds to DNA contains the predicted helix-turn-helix DNA-binding motif

(Zhang et al., 2002). LuxR family proteins likely interact with either the alpha subunit, sigma subunit, or both of RNA polymerase (Egland and Greenberg, 1999; Rhodius and Busby, 1998). Two LuxR residues have been identified as critical for DNA binding, W201 and I206, and replacement of these amino acids with alanine inhibits transcription but does not prevent DNA binding, suggesting that these residues are also critical for activation (Egland and Greenberg, 2001).

2.4. The Basic Molecular Scheme for AHL-mediated QS

Now that we have defined the basic components of AHL-mediated quorum sensing, it is important to describe their interaction and roles. At low cell densities, AHL synthases produce AHL at a low basal level (Nealson, 1977). Because AHL molecules are freely diffusible, AHL diffuses down the concentration gradient into the environment (Kaplan and Greenberg, 1985). As cell numbers increase, the local concentration of AHL builds to a critical threshold, at which point the AHL binds to a LuxR family member inside the cell. In *V. fischeri*, the AHL/LuxR complex binds to a "*lux* box" in the promoter region of the regulated genes (Devine et al., 1989). The *lux* box is a region of dyad-symmetry located upstream of the –35 region for many promoter regions (Stevens and Greenberg, 1999; Zhu and Winans, 1999). LuxR is then thought to activate transcription of quorum sensing–controlled genes by directly interacting with RNA polymerase (Fuqua and Greenberg, 2002; Fuqua et al., 2001).

2.5. QS as a Global Regulatory System

Global AHL-mediated control of gene expression has been most rigorously investigated for *Pseudomonas aeruginosa*. *P. aeruginosa* is an abundant organism in nature, easily isolated from soil and water, and is an opportunistic human pathogen that can cause nosocomial infections as well as chronic infections in the airways of cystic fibrosis patients (Van Delden and Iglewski, 1998). Many *P. aeruginosa* genes linked to virulence (e.g., *lasB*, *lasA*, *aprA*, and *toxA*) are controlled by quorum sensing, and quorum-sensing mutants show reduced virulence in animal models of infection (Gambello et al., 1993; Passador et al., 1993; Seed et al., 1995; Toder et al., 1991, 1994; Whiteley et al., 1999).

There are two AHL signaling systems in *P. aeruginosa*. The LasI/LasR and RhlI/RhlR systems are reasonably well characterized and are known to have interdependence. For instance, transcription of *rhlI* and *rhlR* are induced by the *las* system (de Kievit et al., 2002). A third LuxR homolog, QscR, has been identified in *P. aeruginosa*; however, its function is poorly understood at present (Ledgham et al., 2003). No "QscI" has been identified; nor is such a LuxI homolog predicted based upon an analysis of the sequenced genome (Chugani et al., 2001). It has been estimated that as

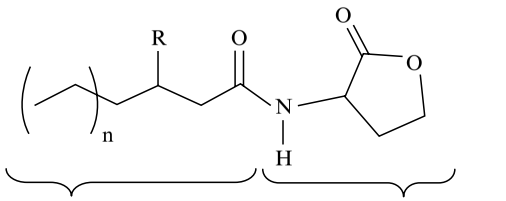
many as 4–6% of the roughly 6,000 *P. aeruginosa* genes are controlled by AHL quorum sensing (Arevalo-Ferro et al., 2003; Schuster et al., 2003; Wagner et al., 2003; Whiteley et al., 1999). Variations in DNA-binding target sequences may function to modulate RhlR/LasR/QscR binding to promoter regions, requiring different amounts of signal to affect expression of a particular gene or operon. Additionally, a non-AHL-signaling molecule has also been described for *P. aeruginosa*. The *Pseudomonas* quinolone signal (PQS), 2-heptyl-3-hydroxy-4-quilinone, plays a significant role in controlling expression of some virulence genes in *P. aeruginosa* and is integrated into the AHL quorum-sensing regulatory circuit (McGrath et al., 2004).

2.6. AHL QS Controls Virulence Functions for Many Pathogens

Several Gram-negative pathogens use AHL systems to control virulence. Like *P. aeruginosa*, *Burkholderia cepacia* also infect cystic fibrosis patients, leading to "cepacia syndrome" (Lewenza et al., 1999). The CepI/R quorum-sensing proteins of *B. cepacia* have been linked to control of virulence, as CepR can function both as a positive (increasing protease and lipase activity) and a negative (decreasing production of the siderophore ornibactin) regulator of extracellular virulence factor production (Lewenza et al., 1999). Two plant pathogens also control virulence gene expression by quorum sensing—*A. tumefaciens* and *Erwinia carotovora* (Miller and Bassler, 2001; Newton and Fray, 2004). *A. tumefaciens* transfers the Ti plasmid to plant cells, producing crown gall tumors. Infected plants are directed to produce opines, which are used as a carbon and nitrogen source by the bacterium. The TraI/R proteins of *A. tumefaciens* are encoded on the Ti plasmid with other factors that promote tumor formation. AHL signaling is used to sense *A. tumefaciens* subpopulations bearing the Ti plasmid (Newton and Fray, 2004). Quorum sensing stimulates conjugal plasmid transfer of the Ti plasmid to plasmid minus subpopulations. *E. carotovora*, uses the CarI/R system to regulate carbapenem antibiotic production, which is believed to play a role in preventing simultaneous infection with other bacteria upon colonization of host plants (Welch et al., 2000; Whitehead et al., 2002). A number of other pathogens that use AHL-based signaling are listed in Table 1.

P. aeruginosa is the pathogen with the best-characterized AHL signaling system. Several factors linked to virulence are under control of one or more of the *P. aeruginosa* quorum-sensing systems. The following functions are quorum sensing–controlled: LasA and LasB are zinc metalloproteases that cleave host biomolecules like elastin and collagen, and AprA is believed to act in the extracellular processing of proteases, including LasA and LasB (Gambello et al., 1993; Morihara and Homma, 1985; Toder et al., 1994). The *xcp* genes encode the type II secretion apparatus involved in secretion of

TABLE 1. AHL molecule structure and function for Gram-negative bacteria.



Bacteria	Regulators	Chain length (n)[b]	$^{\beta}$R-group	Target function	Reference
Vibrio fischeri	LuxR-LuxI	6 (1)	=O	Bioluminescence	Eberhard et al. (1981),
	AinR-AinS[a]	8 (2)	H	Bioluminescence	Gilson et al. (1995), Kuo et al. (1994)
Pseudomonas aeruginosa	LasR-LasI	12 (4)	=O	Host interaction	Pearson et al. (1994, 1995),
	RhlR-RhlI	4 (0)	H	Rhamnolipids	Winson et al. (1995)
	QscR	?	?	Host interaction	
Aeromonas salmonicida	AsaR-AsaI	4 (0)	H	Serine protease	Swift et al. (1997)
Agrobacterium tumefaciens	TraR-TraI	8 (4)	=O	Conjugal transfer	Zhang et al. (1993)
Brucella melitensis	?	12 (0)	H	Regulation of *virB* expression	Taminiau et al. (2002)
Burkholderia cepacia	CepR-CepI	8 (0)	H	Ornibactin, protease, and lipase	Lewenza et al. (1999)
Chromobacterium violaceum	CviR-CviI	6 (0)	H	Pigment production	McClean et al. (1997)
Enterobacter agglomerans	EagR-EagI	6 (0)	=O	Unknown	Swift et al. (1993)
Erwinia carotovora	CarR-CarI	6 (0)	=O	Carbapenem antibiotic production	Welch et al. (2000), Whitehead et al. (2002)

Pantoea stewartii	EsaR-EsaI	6 (1)	=O	Exopolysaccharide	Bainton et al. (1992), Beck von Bodman and Farrand (1995), Swift et al. (1993)
Pseudomonas aureofaciens	PhzR-PhzI	6 (0)	H	Phenazine antibiotic production	Wood and Pierson (1996)
Ralstonia solancearum	SolR-SolI	6 (0), 8 (0)	H, H	Unknown	Flavier et al. (1997a)
Rhizobium leguminosarum	CinR-CinI	14 (5)	OH	Self-inhibition of growth	Gray et al. (1996), Lithgow et al. (2000), Schripsema et al. (1996)
Serratia liquefaciens	SwrR-SwrI	6 (0), 4 (0)	H	Swarming motility	Eberl et al. (1996)
Rhodobacter sphaeroides	CerR-CerI	14 (5)	H	Cell aggregation	Puskas et al. (1997)
Sinorhizobium meliloti	SinR-SinI	12 (0), 14 (0), 16 (5), 16 (5), 18 (0)	H, =O, =O, H, H	Exopolysaccharide and numerous other proteins	Chen et al. (2003), Marketon et al. (2002)
Vibrio anguillarum	VanR-VanI	10 (3)	=O	Proteases	Milton et al. (1997, 2001)
	VanN-VanM[a]	6 (0), 6 (0)	–OH, H	Signal regulation	
Vibrio harveyi	LuxL-LuxM[a]	4 (0)	H	Bioluminescence	Cao and Meighen (1989)
Yersinia enterocolitica	YenR-YenI	6 (1)	H, =O	Unknown	Throup et al. (1995)
Yersinia pseudotuberculosis	YpsR-YpsI	6 (0), 6 (0)	=O, H	Cell clumping and motility	Atkinson et al. (1999)
	YtbR-YtbI	8 (0), 6 (0)	H, H		

[a]AHL synthesis protein-not homologous to LuxI-family proteins
[b]Corresponds to n-position in diagram

LasA and LasB from the cell (Tommassen et al., 1992). Exotoxin A is encoded by *toxA*, which inhibits host cell protein synthesis (Wick et al., 1990). Rhamnosyltransferases produced by *rhlA* and *rhlB* are required for production of rhamnolipids, which are biosurfactants that have cytolytic activity (McClure and Schiller, 1992; Pearson et al., 1997). A stationary-phase sigma factor, σ^S, which regulates various stress-response genes (Pearson et al., 1997; Whiteley et al., 2000). Production of the phenazine pigment pyocyanin, which causes multiple toxic effects in mammalian cells (Chugani et al., 2001; Lau et al., 2004; Ledgham et al., 2003; Whiteley et al., 2000). The *hcnABC* operon controls the synthesis of hydrogen cyanide, a virulence factor that is an inhibitor of cytochrome *c* oxidase, important in aerobic respiration (Pessi and Haas, 2000). LasR controls *lasB*, *lasA*, *toxA*, *aprA*, *xcpP*, and *xcpR* (Van Delden and Iglewski, 1998). RhlR controls expression of *rhlA*, *rhlB*, *lasA*, *rpoS*, *aprA*, *xcpP*, *xcpR*, *phz*, and *hcn* (Van Delden and Iglewski, 1998; Whiteley et al., 1999). QscR has been shown to negatively regulate *lasB* and two *phz* gene clusters and further investigation may show control of other virulence factors (Chugani et al., 2001; Ledgham et al., 2003) (see Figure 1 and Table 1).

3. Non-AHL QS Systems in Gram-negative Species

3.1. The Plant Pathogen Ralstonia solanacearum Utilizes a Volatile Signaling Molecule to Regulate Virulence

R. solanacearum is a phytopathogen shown to cause wilting in a variety of vascular plants, including economically important tomato, tobacco, and peanut species (Schell, 2000). A variety of virulence factors are produced by *R. solanacearum* that contribute to colonization of host plants and cause disease symptoms. Important virulence factors include a high–molecular weight exopolysaccharide (EPS I) and several secreted plant cell wall–degrading enzymes (Schell, 2000). Plant model studies suggest that EPS I causes wilting by blocking the plant's vascular system (Denny, 1995; Denny et al., 1990). A LysR-type DNA-binding regulatory protein, PhcA, controls regulation of many of these virulence factors (Schell, 2000). PhcA both activates and represses expression of different virulence genes (Brumbley and Denny, 1990; Brumbley et al., 1993). PhcA also represents a key intersection point for the input of multiple regulatory systems that modulates expression of virulence functions (Brumbley et al., 1993). Quorum sensing is complex in *R. solanacearum* as multiple signal molecules are produced (Schell, 2000). *R. solanacearum* also possesses a traditional AHL system, encoded by the *luxI* and *luxR* family members, *solI* and *solR*, which probably regulates expression of functions important during late stages of disease (Flavier et al., 1997a). The expression of *solI*/*solR* is controlled by PhcA (Flavier et al., 1997a). However, only a

FIGURE 2. The *Ralstonia solanacearum* quorum-sensing signal molecule, 3-hydroxypalmitic acid methyl ester.

single gene, *aidA*, of unknown function—not present in all *R. solanacearum* strains—is known to be regulated by *solI*/*solR*.

Levels of active PhcA protein are controlled by 3-hydroxypalmitic acid methyl ester (3-OH PAME), a unique volatile signal molecule produced by *R. solanacearum* (Flavier et al., 1997a). The *phcB* gene encodes an enzyme that directs the synthesis of 3-OH PAME (Flavier et al., 1997b). When extracellular 3-OH PAME concentrations are low, *phcA* expression is repressed by the *phcS*/*phcR* two-component system (Clough et al., 1997). However, when 3-OH PAME concentrations reach ~5 nM, *phcA* expression is induced, presumably due to the interaction of 3-OH PAME with *phcS* and subsequent relief of *phcR*-mediated repression of *phcA* (Clough et al., 1997). This signal can act in the gas phase—when spotted on the lid of a petri dish it can activate expression of reporter strains present on the solid growth medium below (Flavier et al., 1997b). Quorum-sensing control using signaling molecules present in the gas phase is exciting, and probably ecologically significant. For example, volatile signals would allow for coordination of gene expression by quorum sensing in nonliquid environments like unsaturated soils. The 3-OH PAME system may represent the first of a new family of compounds that can mediate long-distance intercellular communication (Flavier et al., 1997b) (Figure 2).

3.2. *Myxococcus xanthus and Other Myxococcus species Use QS to Form Fruiting Bodies*

Myxococcus species are commonly found in environmental niches where they feed as a group on dead plant material or other microbial species. *Myxococcus* species are able to utilize complex substrates (compared to most bacteria) including proteins, microbial cell wall components, cellulose, and other complex polymers (Dworkin and Kaiser, 1985). *M. xanthus* is a model species for studying complex multicellular behavior. These bacteria exhibit two types of multicellular behavior: sporulation and gliding motility—a type of social motility (Dworkin and Kaiser, 1985). Spore formation by *M. xanthus* requires a minimum critical mass of ~100,000 individual cells and is a response to starvation on solid surfaces (Dworkin, 1963; Manoil and Kaiser, 1980; Wireman and Dworkin, 1975). Cells develop into spores in the center of a fruiting body—a complex multicellular structure that is formed through the coordinated expression of numerous genes within the population (Shimkets, 1999). Because fruiting-body development requires the presence of such a large

number of cells, *M. xanthus* uses a quorum-sensing mechanism to assess if a sufficient number of starved cells are present to initiate fruiting-body development (Kuspa et al., 1992a,b). Therefore, a specific set of genes expressed early during the development of fruiting bodies is under control of a cell density–dependent signaling mechanism. The signaling molecules are called A-signals (Kuspa et al., 1992b). They are a specific subset of amino acids that function as signals at micromolar to millimolar concentrations, depending upon the amino acid (Kuspa et al., 1992a). These amino acids can also serve as a carbon source to a starved population (Kuspa et al., 1992a). The cells coordinate the arrest of cell growth at the onset of fruiting-body formation. This presumably allows the diversion of the limited energy derived from the amino acids away from growth-related functions to developmental functions.

The A-signals are generated through the activity of secreted or cell surface–associated proteases (Kuspa et al., 1992a). A number of genes have been identified that contribute to *Myxococcus* A-signal generation, including a two-component family histidine kinase–response regulator pair, *asgA* and *asgD*, and a gene encoding a probable DNA-binding protein, *asgB* (Cho and Zusman, 1999; Li and Plamann, 1996; Plamann et al., 1994, 1995). Another pair of two-component family histidine kinase–response regulator homologs, *sasS* and *sasR*, are involved in the sensing of A-signal (Guo et al., 2000). The phosphorylated form of SasR modulates expression of A-signal-controlled genes. In addition, another gene, *sasN*, must be inactivated for signal sensing to occur. The *sasN* gene encodes a protein with no homology to other known proteins and its mechanism remains unknown (Xu et al., 1998).

4. Peptide-based QS in Gram-positive Bacterial Species

4.1. Many Gram-positive QS Systems Use Peptide Signals

Peptide-based quorum sensing in Gram-positive bacteria follows a basic scheme (Figure 3). Precursor signal proteins are ribosomally synthesized within the cell and are subsequently cleaved or processed to form the active signal peptide (Dunny and Leonard, 1997; Miller and Bassler, 2001). The signal peptides are then exported from the cell in an ATP-dependent process by an ABC transporter (Dunny and Leonard, 1997). In some cases, the export of signal across the membrane may coincide with signal processing. The quorum sensing principle is the same as in Gram-negative bacteria—the cell ultimately senses and responds to a buildup of the extracellular signal concentration. However, unlike most Gram-negative AHL signals, the peptide signal does not freely diffuse across the cell membrane (Dunny and Leonard, 1997). The peptide signal is most often sensed at the cell surface by a dedicated receptor protein that is usually a histidine kinase sensor. Such histidine kinase sensors belong, in general, to the well-studied two-component regulatory systems (Miller and Bassler, 2001). The part of the interaction of signal peptide with the sensor kinase results in autophosphorylation of the sensor, which then

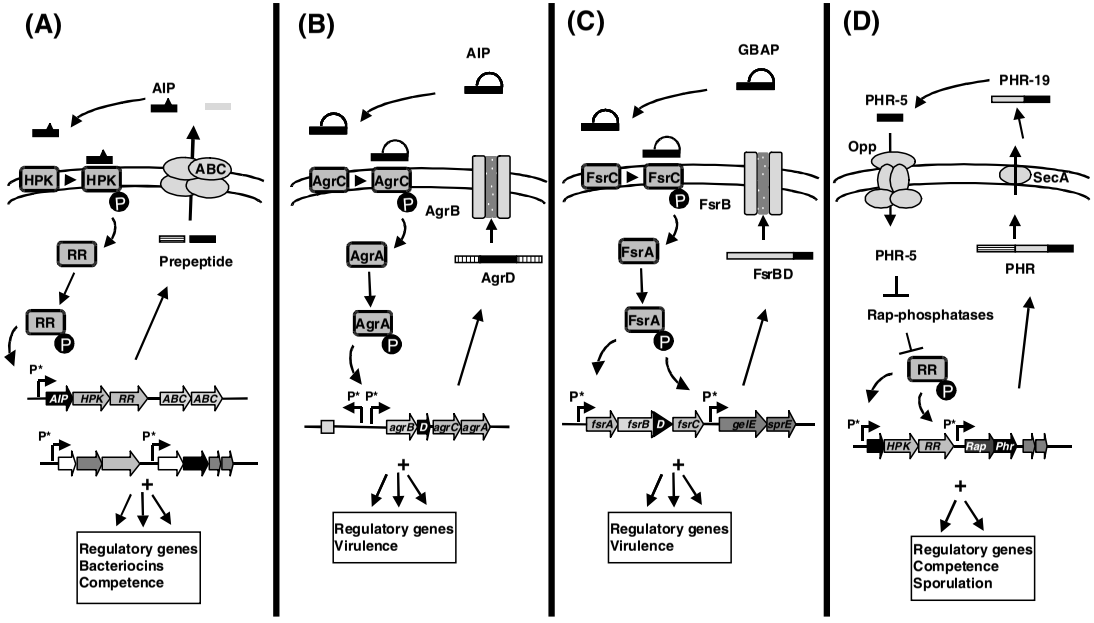


FIGURE 3. Quorum sensing in Gram-positive bacteria. P* indicates regulated promoters in each system. (A) General system for Gram-positive bacteria. HPK: histidine protein kinase; RR: response regulator; ABC: ABC transporter-system; P: phosphate. (B) Staphylococcal *agr*-system. AgrA: response regulator; AgrB: putative AIP-processing/transporter protein; AgrC: histidine protein kinase; AgrD: AIP prepeptide. (C) *Enterococcus faecalis fsr*-system. FsrA: response regulator; FsrB: putative GBAP-processing/transporter protein; FsrBD: FsrB-GBAP fusion protein; FsrC: histidine protein kinase; GBAP: gelatinase biosynthesis-activating pheromone; gelE: gelatinase gene; sprE: serine protease gene. (D) Phr-peptide regulatory system of *Bacillus subtilis*. Opp: oligopeptide-permease; SecA: SecA-dependent transport system; PHR: Phr-protein; PHR-19: Phr-prepeptide; PHR-5: Phr-pentapeptide; Rap: Rap-phosphatase. (From Sturme et al., 2002. Reprinted with permission of Springer Science & Business Media.)

transfers the phosphoryl group to a cytoplasmic, DNA-binding, two-component system response regulator. The phosphorylated form of the response regulator then modulates expression of quorum sensing–regulated genes. As in AHL-based quorum sensing, the production of signal, the two-component sensing machinery, and the ABC transporter are often positively autoregulated.

There are a number of variations to this basic template for peptide-based quorum sensing. The peptide-based *phr* signaling system of *Bacillus subtilis* is an example of a significant deviation from the basic scheme (McQuade et al., 2001). This system is used to regulate competence and sporulation in *B. subtilis* using Phr-pentapeptides (Dunny and Leonard, 1997). Phr signal precursors are ribosomally synthesized in the cytoplasm where they are transported out of the cell by a SecA-dependent general secretory system (Jiang et al., 2000; Simonen and Palva, 1993). Phr precursors are cleaved during transport and, for PhrA, a 19-amino acid peptide results (Perego and Hoch, 1996). PhrA is further processed extracellularly to form a pentapeptide (ARNQT), which functions as the active signaling molecule (Perego, 1997). Instead of acting at the cell surface as seen in other peptide-based systems, the pentapeptide signal enters the cell through the Opp oligopeptide permease system (Perego and Hoch, 1996). Inside the cell, PhrA-pentapeptide inactivates a phosphatase, which results in an accumulation of the active, phosphorylated form of a DNA-binding response regulator. Similar pentapeptide signals are known to be generated for PhrC (ERGMT), PhrE-1 (SRNVT), and PhrE-2 (HEFLV) (Jiang et al., 2000). Although this system represents one of the more extreme variations of peptide-based quorum sensing, it emphasizes the point that variations on the theme are not uncommon.

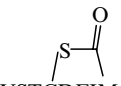
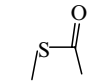
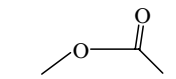
4.2. Signal Peptide Structures Are Highly Variable.

Signal peptides tend to be small, very stable, and subject to posttranslational modifications (Dunny and Leonard, 1997; Miller and Bassler, 2001). The primary roles of these modifications are thought to affect activation and stability of the signaling molecule. The peptide signals can be linear or cyclic, ranging from 5 to 26 amino acid residues (see Table 3) (Dunny and Leonard, 1997; Federle and Bassler, 2003). After a precursor peptide is ribosomally synthesized, it is processed during transport (Sturme et al., 2002). Depending upon the system, the peptide can also be processed before and/or after transport (Sturme et al., 2002). Besides proteolytic cleavage, peptide signals can be posttranslationally modified in a number of fashions (Sturme et al., 2002). The signal peptides of *Lactococcus lactis* and *B. subtilis* have been shown to contain dehydrated amino acids and amino acids with thioether ring structures such as lanthionine or beta-methyl-lanthionine (Sturme et al., 2002). These modifications are introduced into the signal peptide by cytoplasmic enzymes prior to transport. There are numerous systems that use linear signal peptides, such as ComC of the human respiratory pathogen *S. pneumoniae*

(Håvarstein et al., 1995) and PlnA of *Lactobacillus plantarum* (Dunny and Leonard, 1997), that are not modified except for cleavage of the peptide leader sequence during transport at a characteristic double-glycine residue motif.

The pathogens *Staphylococcus aureus* and *Enterococcus faecalis* produce cyclic signal peptides (Bassler, 2002; Dunny and Leonard, 1997; Mayville et al., 1999; Nakayama et al., 2001). *S. aureus* signals contain a thio-lactone ring (see Table 3), while the gelatinase biosynthesis-activating pheromone (GBAP) signal of *E. faecalis* harbors a lactone ring (which is similar to a thiolactone ring, where the sulfur atom is substituted with an oxygen atom) (Nakayama et al., 2001). The introduction of these ring structures is thought to occur during the export process (Sturme et al., 2002).

TABLE 2. Specific structures of peptide signal molecules[a]

Organism	Name of signal	AA Sequence of mature molecule	Length of unprocessed precursor (AA)
Lactococcus lactis	Nisin A	ITSISLCTPGCKTGALMG CNMKTATCHCSIHVSK[b]	57
Lactobacillus plantarum	IF (PlnA)	KSSAYSLQMGATAI KQVKKLFKKWGW	48
Streptococcus pneumoniae	CSP (ComC)	EMRLSKFFRDFILQRKK	41
S. gordonii	CSP (ComC1)	DVRSNKIRLWWENIFFNKK	50
Bacillus subtilis	ComX pheromone	ADPITRQW[c]GDERGMT	55
	CSF (PhrC)		40
Staphylococcus aureus	AgrD	 YSTCDFIM GVNACSSLF	44
		 INCDFLL YSTCYFIM	
Enterococcus faecalis	cCF10	LVTLVFV	Not determined
	iCF10	AITLIFI	23
	cAD1	LFSLVLAG	Not determined
	iAD1	LFVVTLVG	23
	GBAP	 QNSPNIFGQWM	242

[a]Adapted from Dunny and Leonard 1997; Bassler 2002; Federle and Bassler 2003.
[b]Multiple posttranslational modifications. Nisin has extensive posttranslational modifications of several amino acid residues. AgrA appears to contain a cyclic anhydride structure.
[c]ComX pheromone is beleived to have a modified tyrosine residue.

4.3. Some Signal Peptides also Function as Bacteriocins

Alternative functions for peptide signals have been identified in some bacterial species. In addition to cell signaling, a small subgroup of peptide signals has also been shown to have antimicrobial activity (Hechard and Sahl, 2002). Perhaps the best-studied system of this subgroup of signals is nisin, produced by *L. lactis* (Sturme et al., 2002). Nisin is an antibiotic belonging to class I (modified peptides) antimicrobial peptides (Sturme et al., 2002). Nisin uses lipid targets on bacterial membranes as docking molecules for formation of highly effective targeted pores (Gao et al., 1991). Disruption of bacterial membranes by nisin can also occur in a nonspecific, target-independent manner (Hechard and Sahl, 2002). Subtilisin of *B. subtilis* is another example of a class I antimicrobial peptide that can function as a signaling molecule (Sturme et al., 2002). There are also class II (unmodified peptides) antimicrobial peptides, called bacteriocins, that function as signaling peptides. Two examples are the linear peptides carnobacteriocin of *Carnobacterium piscicola* LV17B and Plantaracin A of *L. plantarum* C11 (Hechard and Sahl, 2002). Class II bacteriocins usually function by forming pores in the membrane and dissipating proton motive force (Hechard and Sahl, 2002). The dual functionality of these peptides is thought to serve a group of bacteria trying to establish an ecological niche in a competitive environment.

4.4 QS Controls Competence in Streptococcus pneumoniae

The Com system of *S. pneumoniae* is required to achieve a competent state, which allows uptake of extracellular DNA. This discovery was actually the first report of quorum sensing for bacteria (Tomasz, 1965). The competent state is only temporary and occurs in late logarithmic growth in liquid batch culture (Tomasz, 1965). How the cell shuts off the quorum-sensing response is not clear.

The Com quorum-sensing system operates in a cascade of steps. The 41 amino acid peptide ComC is cleaved posttranslationally to the 17 amino acid competence stimulating peptide (CSP) which is then secreted by the ABC transporter, ComAB (Håvarstein et al., 1995). Ultimately, at high cell densities, CSP reaches a critical concentration at which it interacts with the histidine kinase ComD (Whatmore et al., 1999). ComD is autophosphorylated and subsequently transfers its phosphate group to the response regulator ComE (Whatmore et al., 1999). The phosphorylated ComE induces expression of the alternative sigma factor, ComX, which in turn is responsible for modulating expression of a subset of genes in the *S. pneumoniae* quorum-sensing regulon (Peterson et al., 2004). Recent microarray studies have determined that a large number of ComX-independent genes appear to be regulated by quorum sensing, including some whose function appears to be unrelated to competence (Peterson et al., 2004).

4.5. Staphylococcus aureus *Virulence is controlled by QS*

S. aureus can cause endocarditis, septic arthritis, and toxic shock syndrome. A number of virulence factors of this opportunistic human pathogen appear linked to a peptide-based quorum-sensing system (see Novick, 2003 for a recent review). The accessory gene regulator (*agr*) system of *S. aureus* controls both secreted and cell-associated virulence factors and has been shown to be required for infection in a murine model (Mayville et al., 1999).

This system consists of the two-component histidine kinase–response regulator pair, AgrC/AgrA, the membrane localized transport and processing enzyme, AgrB, and the signal pro-peptide, AgrD (Podbielski and Kreikemeyer, 2004; Yarwood and Schlievert, 2003). AgrD is processed post-translationally at both the amino and carboxy terminus to form the active signal peptide (Lina et al., 1998). The signal peptide consists of 7–9 amino acids (depending upon the system) and contains a thio-lactone ring involving an internal conserved cysteine and the carboxylate group of the peptide's carboxy-terminal amino acid (Ji et al., 1995). The last component of the *agr* system is the regulatory RNA, RNAIII. RNAIII production is thought to be induced by binding of AgrA to the P3 promoter (Lina et al., 1998). AgrA can also induce expression of the *agrBDCA* operon, resulting in positive autoregulation of quorum sensing (Podbielski and Kreikemeyer, 2004). It is through RNAIII that the *agr* system exerts its global effects upon gene expression (Xiong et al., 2002). RNAIII is highly abundant in the cell with extensive secondary structure and a very long half-life (over 15 min) (Benito et al., 2000; Janzon and Arvidson, 1990). The mechanism by which RNAIII acts is unclear, although it has been suggested that it may bind to key regulatory proteins or interfere with the translation of regulatory proteins by mimicking Shine–Dalgarno sequences (Novick et al., 1993).

In summary, the basic scheme for *S. aureus* quorum sensing is thought to be the following: AgrD is processed and secreted by AgrB. At high cell densities, the signal peptide binds extracellularly to AgrC, which autophosphorylates itself and then transfers a phosphoryl group to AgrA. Phosphorylated AgrA then binds to the P3 promoter to induce RNAIII synthesis.

There have been four *agr* specificity groups that have been defined in *S. aureus* strains (Ji et al., 1997). The specificity group is characterized by the ability of peptide signals produced by strains from one group to inhibit the quorum-sensing mechanism of the other three groups through competitive binding of the signal to the histidine kinase sensor AgrC (Lina et al., 1998). Although the specificity of these four groups primarily occurs at the level of the peptide signal–AgrC interaction, there is some specificity of AgrB for AgrD within the four groups (Jarraud et al., 2000; Ji et al., 1997). The different specificity groups have been implicated in different aspects of *S. aureus* pathogenesis. For example, most menstrual toxic shock syndrome strains belong to *agr* specificity group II (McCormick et al., 2001).

5. Nonpeptide-based QS Systems in Gram-positive Species

5.1. The A-factor of Streptomyces *Species Is a γ-butyrolactone Signal*

Not to be confused with A-factor of *Myxococcus* species, A-factor (2-isocapryloyl-3R-hydroxymethyl-γ-butyrolactone) of *Streptomyces griseus* is required for both morphological differentiation and production of extracellular compounds (Ohnishi et al., 1999). *Streptomyces* species are soil bacteria that show complex morphological development—bacteria form spores that germinate into branched, filamentous, multinucleodial hyphae (Ohnishi et al., 2002). Aerial mycelium formation, important to hyphal branching, as well as streptomycin production, streptomycin resistance, yellow pigment production, and melanin-like molecule production, are all dependent upon A-factor quorum sensing in *S. griseus* (Natsume et al., 2004; Ohnishi et al., 2002).

A-factor is believed to be synthesized by AfsA (Ohnishi et al., 2002). Once synthesized, A-factor diffuses into the local environment and, at sufficient concentrations, binds to an A-factor-specific receptor, ArpA (Ohnishi et al., 1999). The only *Streptomyces* A-factor receptor protein structure determined to date is CrpB of *S. coelicolor* (Natsume et al., 2004). CrpB, an ArpA homolog, is a two-subunit protein composed of a DNA-binding domain and a regulatory domain (Natsume et al., 2004). Binding of A-factor to ArpA releases bound AdpA, a transcriptional activator, which controls expression of several *Streptomyces* quorum sensing–regulated genes. Bacteria with *adpA* disruptions cannot produce either streptomycin or yellow pigment, and are "bald" without hyphae (Ohnishi et al., 1999, 2002). Currently, AdpA is also known to activate transcription of several genes including *amfR* (involved in synthesis of an extracellular peptidic morphogen), *adsA* (encodes an ECF sigma factor), and *sgmA* (involved in synthesis of extracellular proteases) (Ohnishi et al., 2002) (see Figure 3 and Table 2).

6. AI-2 QS

6.1. One Form of Interspecies QS Utilizes a Signal Called AI-2

In the last 10 years, exciting new information has emerged indicating that some bacteria can sense QS signals produced by other species (Federle and Bassler, 2003; Taga and Bassler, 2003; Taga et al., 2003). AI-2 signaling appears to allow interspecies quorum sensing as many species have a gene called *luxS*, required for synthesis of AI-2 signal (Surette et al., 1999). AI-2 can also be used for intraspecies signaling. AI-2 signaling was first described

for *V. harveyi*, which uses both AI-2 and another signal, AI-1 (an AHL produced by LuxM, see Table 2), to regulate bioluminescence (Surette et al., 1999). AI-2 and AI-1 of *V. harveyi* have also been shown to independently regulate genes involved in other functions. The prevalence of LuxS in many Gram-positive and Gram-negative bacterial species supports the idea that AI-2- signaling is widespread. Homologs of the *luxS* gene have been identified in at least 55 sequenced bacterial genomes and other species whose genomes have yet to be sequenced (Taga and Bassler, 2003).

6.2. *AI-2 Is a Furanosyl Borate Diester that Binds with Two Proteins to Initiate a Signaling Cascade*

Similar to AHL signals, SAM serves as a precursor for AI-2 biosynthesis in *V. harveyi* (Chen et al., 2002). The first step of AI-2 synthesis involves demethylation of SAM to form S-adenosylhomocysteine (SAH), which is then converted to S-ribosylhomocysteine (SRH) and adenine (a by-product) through hydration by the enzyme Pfs in *V. harveyi* (Chen et al., 2002). LuxS acts to release homocysteine from SRH to form 4,5-dihydroxy-2,3-pentanedione (DPD) (Schauder et al., 2001). DPD appears to be the common precursor for at least two chemically distinct AI-2 molecules. While it is known that the AI-2 of *V. harveyi* contains boron, AI-2 of *Salmonella typhimurium* does not (Miller et al., 2004). It is currently believed that DPD spontaneously rearranges into several furanone structures, which are further modified to active AI-2 molecule(s) (Figure 4). (The use of boron in AI-2 may explain the previous mystery of its requirement as an essential element for some species; Federle and Bassler, 2003.)

Response to AI-2 signal in *V. harveyi* requires the proteins LuxP and LuxQ (Federle and Bassler, 2003). LuxP is a soluble periplasmic protein with high similarity to RsbB (a ribose recognition protein) of *Escherichia coli* and *S. typhimurium* (Chen et al., 2002). LuxP is believed to have nine amino acid residues that interact with AI-2 (Q77, S79, W82, N159, R215, D267, T266, W289, and R310) (Chen et al., 2002). The LuxP/AI-2 complex is recognized by a second protein, LuxQ (Federle and Bassler, 2003). LuxQ is a two-component hybrid sensor kinase that spans the inner membrane. The AI-2/LuxPQ complex then initiates a cascade of phosphorylation signaling that results in transcription of regulated genes. AI-2-dependent transcriptional activation has been determined for *Shigella flexneri* (Xavier and Bassler, 2003), and discovery of additional AI-2 responsive transcription factors is a current area of research as such regulation has been predicted but not yet verified. In *V. harveyi*, AI-2/LuxPQ acts to dephosphorylate LuxO (at residue H2), which subsequently dephosphorylates LuxU (at residue D2) allowing LuxR activation of the *luxCDABE* operon which encodes genes involved in bioluminescence (Federle and Bassler, 2003).

TABLE 3. Bacterial pathogens possessing quorum-sensing systems.

Bacterium	Signal type	Quorum-sensing components	Clinical significance	References
Pseudomonas aeruginosa	AHL	LasI/LasR, RhlI/RhlR, QscR	CF lung infections, Skin burn infections	Fuqua et al. (2001), Parsek and Greenberg (2000)
Agrobacterium tumefaciens	AHL	TraI/TraR	plant pathogen	Fuqua and Greenberg (2002), Fuqua et al. (2001), Miller and Bassler (2001)
Erwinia caratovora	AHL	CarI/CarR, ExpI(/ExpR?)	Carbapenem antibiotics	Fuqua and Greenberg (2002), Miller and Bassler (2001)
Streptococcus pneumoniae	Peptide and AI-2	ComC-ComD/ComE	respiratory tract infection	Dunny and Leonard (1997), Håvarstein et al. (1995), Miller and Bassler (2001)
Staphylococcus aureus	Peptide	AgrD-ArgC/ArgA	respiratory tract, skin, and intestinal infections	Ji et al. (1995), Mayville et al. (1999)
Burkholderia cepacia	AHL	CepI/CepR	CF lung infections	Huber et al. (2001), Lewenza et al. (1999)
Aeromonas hydrophila	AHL	AhyI/AhyR	fish & mammalian pathogen	Lynch et al. (2002), Swift et al. (1997, 1999)
Salmonella typhimurium	AI-2	LuxS, LsrK, LsrR	Typhoid fever Salmonellosis	Taga and Bassler (2003), Taga et al. (2003)
Escherichia coli O157:H7	AI-2	LuxS	Hemorrhagic colitis Hemolytic-uremic syndrome	Sperandio et al. (2001, 2003)
Neisseria meningitidis	AI-2	LuxS	Bacterial meningitis	Winzer et al. (2002)

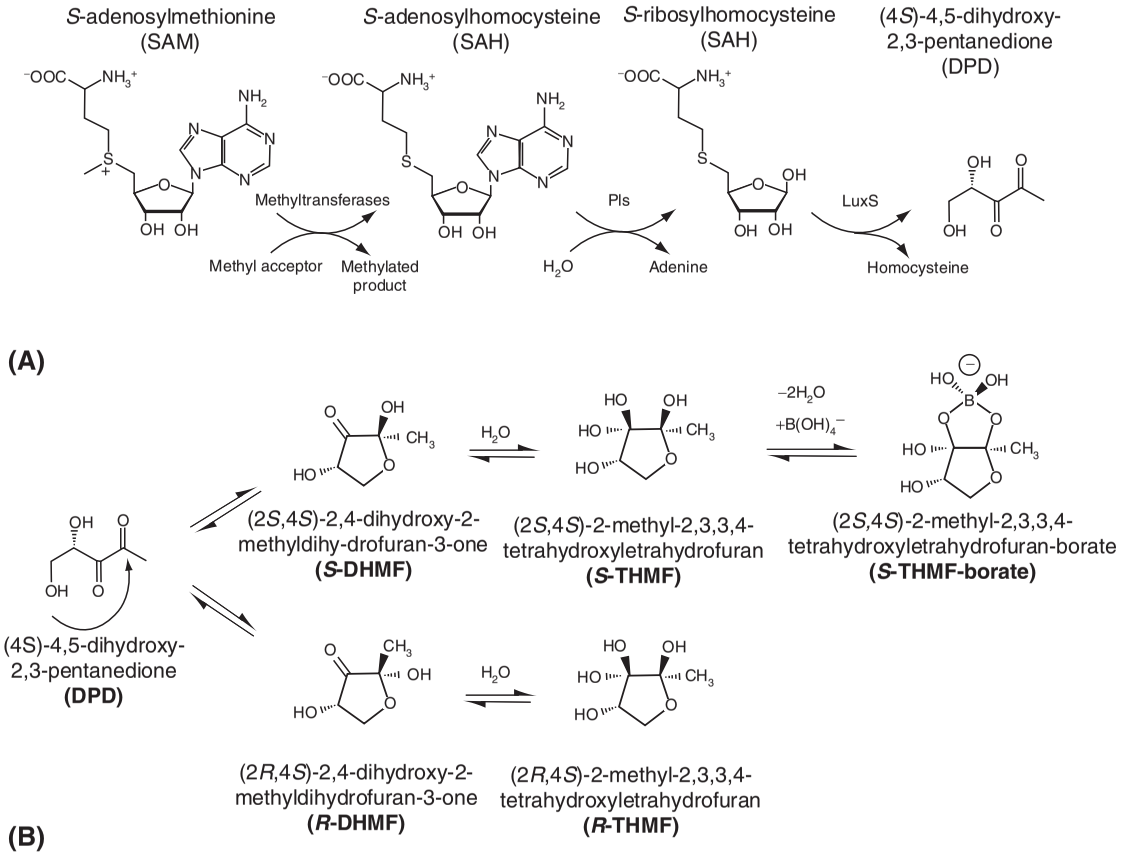


FIGURE 4. Formation of AI-2. (A) Pathway of DPD formation from SAM involving LuxS. (B) Model showing proposed formation of AI-2 for *Vibrio harveyi* (upper pathway) and *Salmonella typhimurium* (lower pathway). (From Miller et al., 2004. Reprinted with permission from Elsevier.)

6.3. AI-2-dependent QS Regulates Virulence Factors in Many Species

AI-2 has already been shown to be recognized across species by a remarkable number of bacteria (Federle and Bassler, 2003). Known responses to AI-2 include enterohemorrhagic and enteropathogenic type III secretion in *E. coli* that assist in attachment to intestinal epithelia (Sperandio et al., 2001, 2003). Virulence gene expression in *Neisseria meningitidis*, *V. cholerae*, *S. flexneri, S. pyogenes*, and *Actinobacillus* species are influenced by AI-2 signaling (Bassler, 2002; Federle and Bassler, 2003; Miller and Bassler, 2001; Winzer et al., 2002). AI-2 is used by *Clostridium perfringens* to control toxin production (α-, κ-, and θ-toxin) related to gas gangrene (Ohtani et al., 2002).

Many pathogenic bacteria have been shown to have *luxS* genes that have not been linked to control of virulence. For instance, in *S. typhimurium*, AI-2 signal has only been linked to activation of an ABC transporter called Lsr (LuxS-regulated) (Taga et al., 2003). AI-2 signaling appears to be involved in the formation of surface-associated communities called biofilms for several bacteria, including *Porphyromonas gingivalis*, *Salmonella typhi*, and *Streptococcus mutans* (Federle and Bassler, 2003); also interesting is the report that *luxS* mutants of *Helicobacter pylori* showed increased biofilm formation (Cole et al., 2004). In the periodontal pathogen *Actinobacillus actinomycetemcomitans*, AI-2 signaling has been linked to both expression of AfuA, an iron transport protein, and expression of leukotoxin, a virulence-associated protein (Fong et al., 2001) (Figure 4).

7. Conclusions

7.1. QS Coordinates Gene Expression through Extracellular Signaling Molecules

Only when the signal reaches a threshold concentration does regulated gene expression proceed. Because signal production is relatively constant for one bacterium, a quorum of bacteria is required to produce local signal concentrations above the threshold.

7.2. Many Gram-negative Species Utilize AHL Signaling Molecules

AHL quorum sensing requires two proteins—a LuxI-type protein to produce the freely diffusible AHL signal molecule and a LuxR-type protein to complex with the AHL signal and subsequently regulate expression of quorum sensing-controlled genes.

7.3. QS in Gram-positive Species Commonly Uses Peptide Signals

Active signal molecules are generated by cleaving translated proteins to form short peptide chains, and for some species these peptides are further modified before they are active signals. The peptide regulates gene expression through a two-component regulatory system.

7.4. QS Controls Virulence Functions in many Bacterial Species

These functions include induction of bioluminescence in *V. fischeri*, activation of some virulence genes in *P. aeruginosa*, and induction of competence in *S. pneumoniae*. Within recent years the ability of QS to control gene expression across bacterial species has also been identified. AI-2 quorum sensing controls virulence factors for *V. cholerae*, *Streptococcus* species, and *S. flexneri* (Table 3).

Questions to Consider

1. What are some of the predominant quorum-sensing signal molecules produced by Gram-negative and Gram-positive species?

Many Gram-negative species are known to produce acyl-homoserine lactone (AHL) molecules for quorum-sensing signaling; Gram-positive species predominantly utilize peptide or modified peptide signals.

2. What are the proteins required for AHL signaling in the model Gram-negative species *Vibrio fischeri*?

LuxI is required for signal production and LuxR is a DNA-binding signal receptor protein.

3. What is the name of the quorum-sensing signal that has been identified for interspecies quorum sensing?

The interspecies quorum-sensing molecule is called AI-2. For *Vibrio harveyi*, this has been determined to be a furanosyl borate diester derived from S-adenosylmethionine.

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Chapter 12

The Role of Sigma Factors in Regulating Bacterial Stress Responses and Pathogenesis

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Historical Landmarks

1969 Sigma subunit of RNA polymerase (σ^{70}) discovered by Burgess et al. (1969).

1978 Travers et al. (1978) discover role of sigma in activation of transcription.

1979 Discovery of first alternative sigma factor in bacteria, σ^{B}, in *Bacillus subtilis* (Haldenwang and Losick, 1979).

1981 Losick and Pero (1981) discover cascade regulation of gene expression in *B. subtilis* by alternative sigma factors.

1984 First alternative sigma factor in *Escherichia coli* discovered (σ^{32}) (Grossman et al., 1984; Landick et al., 1984; Yura et al., 1984).

1984 First report of small RNAs that regulate bacterial gene expression (Mizuno et al., 1984).

1985 First member of σ^{54} family discovered (Hirschman et al., 1985; Hunt and Magasanik, 1985).

1989 Researchers demonstrate recognition of the −10 and −35 promoter regions by σ^{70} (Gardella et al., 1989; Siegele et al., 1989; Zuber et al., 1989).

1989–1999 Identification of sigma regions important for binding to core RNA polymerase (Lesley and Burgess, 1989; Lesley et al., 1991).

1989–1990 Identification of sigma regions important for binding to promoter DNA (Gardella et al., 1989; Siegele et al., 1989; Waldburger et al., 1990).

1992 Fang et al. (1992) report that σ^{38} regulates bacterial virulence.

1996–2003 High-resolution images of RNA polymerase core, holoenzyme, individual sigma domains, as well as sigma complexed to antisigma factors (Campbell et al., 2002, 2003; Li et al., 2002; Malhotra et al., 1996; Murakami et al., 2002a, 2002b; Vassylyev et al., 2002; Zhang et al., 1999).

1. Introduction

1.1. Bacterial Stress Responses and Sigma Factors

Bacterial adaptation and survival requires the ability to sense and respond to a wide range of environmental stresses. This is particularly true for bacterial pathogens, which must respond to a wide variety of host environmental stresses during the course of infection, including changes in nutrient availability, pH, temperature, osmolarity, oxidation, and fluid shear (Audia et al., 2001; Cavicchioli et al., 2000; Foster and Spector, 1995; Hecker and Volker, 2001; Hengge-Aronis, 2002a; Nickerson et al., 2004; Poolman et al., 2002). Many of these environments are less than optimal for bacterial growth and can even

threaten the viability of the organism. Thus, it is not surprising that exposure to these stresses induces global changes in gene expression that allow bacteria to rapidly adapt to, and survive, the new conditions. Bacteria possess a complex network of global regulatory systems, which act as stress sensors and effectors that ensure a coordinated and effective molecular response to changing environmental stimuli (Wick and Egli, 2004). A common mechanism used by bacteria to bring about global changes in gene expression in response to environmental stress is the use of different (i.e., alternative) sigma (σ) factors.

Bacterial sigma factors are a family of proteins that regulate gene expression by interacting with RNA polymerase to direct the initiation of transcription from specific promoter sequences (Burgess and Anthony, 2001; Gruber and Gross, 2003; Haldenwang, 1995; Helmann et al., 2001). The major sigma factor that is responsible for directing general transcription and the bulk of RNA during exponential growth of bacteria (called the "housekeeping," "primary," or "vegetative" sigma factor) is reversibly associated with RNA polymerase and can be replaced by alternative sigma factors. These alternative sigma factors are responsible for regulating the initiation of transcription of specialized sets of genes in response to specific environmental stimuli. Thus, RNA polymerase initiates transcription of different sets of genes depending upon the sigma factor that is bound to it. Most bacteria have multiple alternative sigma factors that they use to coordinately regulate the expression of genes whose products are involved in diverse functions, such as stress responses, morphological development, chemotaxis, and iron uptake. Bacteria with more varied lifestyles, including some pathogens, often contain more alternative sigma factors. This is logical, since organisms that encounter varied environments need to adjust their metabolism and respond to many stresses (often simultaneously), which requires a large repertoire of regulatory mechanisms (Mittenhuber, 2002). For example, *Streptomyces coelicolor*, a Gram-positive antibiotic-producing soil bacterium that undergoes a complex developmental life cycle, has an astonishing 63 sigma factors, the most of any known bacteria (Bentley et al., 2002). The spore-forming bacteria *Bacillus subtilis*, the organism in which the cascade regulation of gene expression by alternative sigma factors was first discovered, has 18 sigmas (Gruber and Gross, 2003; Losick and Pero, 1981). By comparison, the smallest known species of bacteria, the *Mycoplasma (*including the pathogenic *Mycoplasma pneumoniae),* have only 1 sigma factor (Fraser et al., 1995; Himmelreich et al., 1996), whereas the enteric pathogens *Escherichia coli* and *Salmonella typhimurium* have seven (Wick and Egli, 2004), the respiratory pathogen *Mycobacterium tuberculosis* has 13 (Cole et al., 1998; Gomez, 2000; Gomez et al., 1997; Manganelli et al., 2004b), and the opportunistic pathogen *Pseudomonas aeruginosa* has 24 (Mittenhuber, 2002).

This chapter will focus on alternative sigma factors used by bacterial pathogens to respond to environmental conditions relevant to those encountered within the infected host, and how these sigma factors regulate the expression of stress and virulence genes, which allow the organism to persist, replicate, and resist host defenses.

1.2. Bacteria Use Sigma Factors to Activate Transcription of Genes

Sigma factors are required for promoter recognition and initiation of transcription by the bacterial RNA polymerase. The initiation of transcription is the principal step at which bacterial gene expression is regulated. The specificity of the transcription reaction largely relies on the interactions between RNA polymerase (the enzyme that copies the genetic information from DNA to RNA) and the DNA promoter elements with which it makes contact. Bacterial core RNA polymerase is composed of five subunits, α_2, β, β', and ω (Gross et al., 1998), and contains all the enzymatic functions necessary for transcription elongation and termination. However, the core form of RNA polymerase is unable to initiate transcription. To initiate transcription, bacterial core RNA polymerase must associate with a small dissociable subunit, called sigma (σ). The sigma factor reversibly binds to the RNA polymerase to form the holoenzyme ($\alpha_2\,\beta\beta'\,\sigma$), which is then competent to recognize and bind to a specific class of promoter sequences (the sites on DNA where RNA polymerase binds to initiate transcription) (Figure 1). The formation of the RNA polymerase holoenzyme is a crucial step in the initiation of transcription, as neither the core RNA polymerase (i.e., without sigma) nor the sigma subunit alone are able to specifically recognize and bind to the promoter sites that signal the starts of genes. Thus, sigma confers promoter-specific transcription initiation on RNA polymerase and, as such, plays a major role in promoter recognition by the enzyme.

Upon sigma binding to the core RNA polymerase, a conformational change occurs that allows the DNA-binding regions of sigma to become unmasked and bind to the double-stranded DNA in the promoter region to form a "closed complex" (Gross et al., 1998) (Figure 2). In order for transcription to proceed, however, the double-stranded DNA in the promoter region (i.e., closed complex) must be separated (i.e., "untwisted" or "melted") into single-stranded regions to form the "open complex" (Figure 2). Open complex formation, which is also dependent upon sigma, is essential for RNA polymerase to initiate transcription in the presence of nucleoside triphosphates (NTPs). After about 9–12 nucleotides of RNA have been synthesized, conformational changes occur in the holoenzyme that result in sigma dissociation. Sigma dissociation from RNA polymerase is essential for promoter clearance by the enzyme, and allows it to enter into processive elongation and production of a full-length transcript. Thus, in addition to conferring promoter specificity and binding to RNA polymerase, sigma plays a key role in maintaining open complex formation, promoter clearance and escape, and the early stages of transcript elongation (Hernandez et al., 1996; Roberts et al., 1998). However, sigma can only perform these functions when bound to RNA polymerase. Sigma factors are also able to bind to a variety of transcriptional activators and antisigma factors to alter the affinity of RNA polymerase for certain promoters, thus adding another layer of

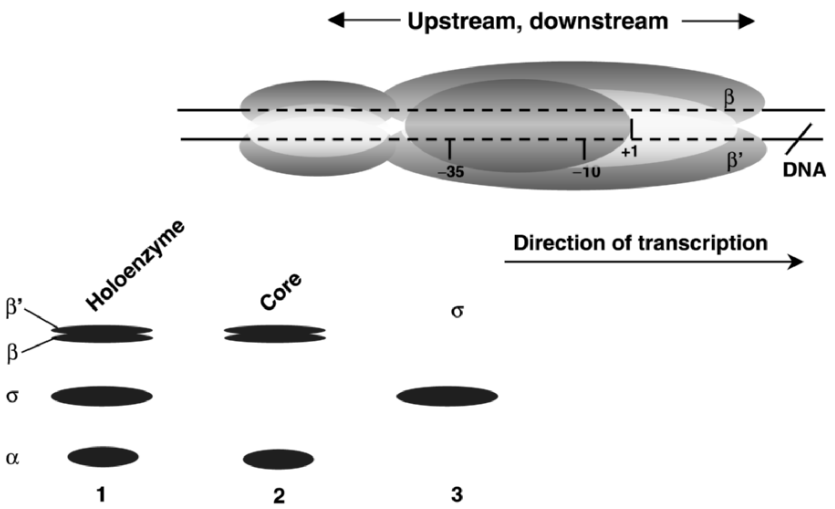

FIGURE 1. The structure of bacterial RNA polymerase. The core enzyme consists of α_2, β, β′, ω, and requires the addition of a separate dissociable subunit, σ, to form the holoenzyme, which can then initiate transcription. (Note: the ω subunit is not shown, as it can be removed from RNA polymerase without affecting any known properties of the enzyme.) The σ^{70}-RNA polymerase holoenzyme is shown bound to the consensus promoter elements –10 and –35.

complexity to the process of transcriptional regulation (Hughes and Mathee, 1998; Russo and Silhavy, 1992). Given its roles in binding core RNA polymerase, promoter DNA, and transcription factors, multiple sites of interaction clearly exist between sigma and these molecular components. Recent high-resolution X-ray crystallographic images of a sigma domain alone and in complex with these different molecular components have provided a wealth of structural information that is in agreement with the functional properties of sigma (Borukhov and Nudler, 2003; Burgess and Anthony, 2001; Gruber and Gross, 2003).

Most transcription in exponentially growing bacteria is initiated by RNA polymerase holoenzyme that contains a housekeeping sigma factor similar to *E. coli* σ^{70} (Gruber and Gross, 2003). Originally identified in *E. coli*, the major housekeeping sigma, σ^{70} (encoded by the *rpoD* gene), is the best characterized of all sigma factors (Burgess and Anthony, 2001; Burgess et al., 1969). All known eubacteria contain a housekeeping sigma that is similar to *E. coli* σ^{70} (referred to as SigA/σA in Gram-positive organisms), which is essential for bacterial growth under normal physiological conditions (Gruber and Gross, 2003). Sigma 70 and its homologs in other bacteria are responsible for the transcription of the majority of bacterial genes during exponential growth, commonly called the housekeeping genes. Housekeeping genes encode products that are important for the major biosynthesis pathways, including assimilation of amino acids and nucleotides, enzyme cofactors, cell

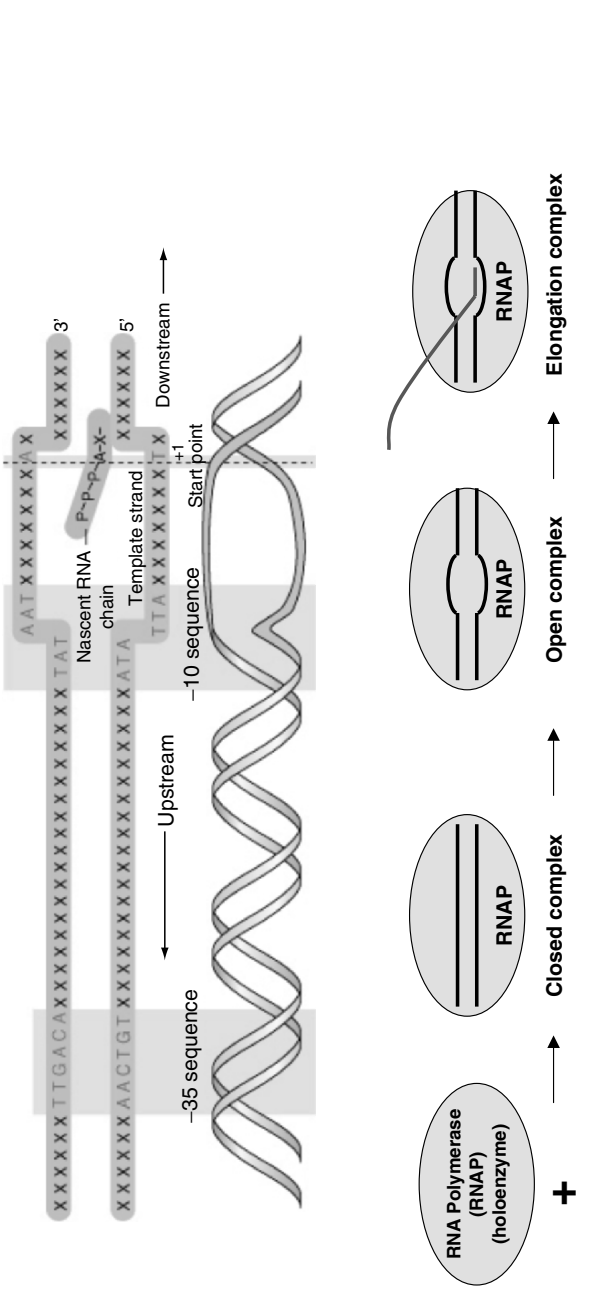

FIGURE 2. RNA polymerase/DNA complexes. Transcription initiation is a multistep process that proceeds through the formation of closed to open promoter complexes (see text for details). (From Karp, Cell and Molecular Biology, © 1999 John Wiley and Sons; reprinted with permission.)

membrane and cell wall components, and carbon utilization enzymes in the glycolysis, pentose phosphate shunt, and TCA cycle (Gross et al., 1992). Sigma 70 is responsible for RNA polymerase, recognizing promoters that have three conserved elements: (1) the –10 site, centered at 10 base pairs (bp) upstream of the start-point of transcription (designated as +1), this sequence element has the consensus sequence TATAAT (Fig. 3); (2) the –35 site, centered at 35 bp upstream of the start-point of transcription, this sequence element has the consensus sequence TTGACA (Fig. 3); and (3) the spacer region, the critical distance between the –10 and –35 sites, which is conserved at 17 ± 1 bp. The consensus sequences of the *E. coli* RNA polymerase σ^{70} promoter were deduced from sequence compilations, and the importance of these conserved promoter elements has been demonstrated by random and site-directed mutagenesis studies (Figure 3) (Collado-Vides, 1993; Record et al., 1996). Most of these studies focused on the effect of promoter sequence changes on transcriptional efficiency.

1.3. Alternative Sigma Factors Activate Transcription of Specialized Gene Sets in Response to Environmental Stimuli

In addition to the major housekeeping sigma factor, σ^{70}, bacteria have alternative sigma factors that regulate the expression of particular gene sets in response to specific environmental conditions. Just like σ^{70}, these alternative sigmas must bind to core RNA polymerase to effect initiation of transcription of different regulons. However, these alternative sigma factors possess different promoter recognition properties as compared to σ^{70}, and usually regulate the expression of genes that are important for bacterial survival dur-

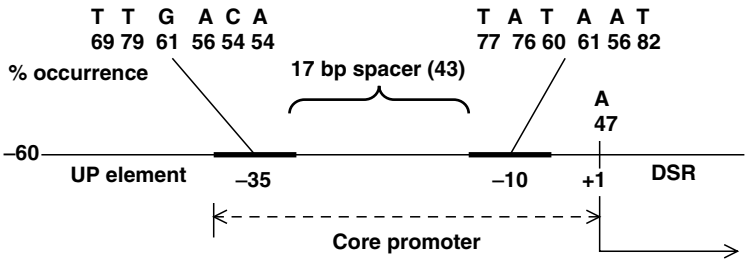

FIGURE 3. Structure of the "consensus" σ^{70} promoter showing functionally important regions. Consensus sequences of the –35 and –10 hexamers and the consensus spacer length shown are correlated with their percentage of occurrence in the database of approximately 300 promoter sequences. The upstream (UP) element and downstream region (DSR) are of functional importance for some σ^{70} promoters. (Adapted from Record et al., 1996.)

ing suboptimal or stressful growth conditions. Because most alternative sigma factors are related to σ^{70} (see below), they direct RNA polymerase to initiate transcription from promoters that contain variations of the conserved σ^{70} promoter consensus sequences. The exception to this rule are members of the σ^{54} family, which share no sequence homology with the σ^{70} family, that direct the RNA polymerase to promoter sequences that are much different than those that are important for recognition by the σ^{70} family. The σ^{54}-containing holoenzyme recognizes a pair of promoter elements that are located at –12 and –24 with respect to the start of transcription that have the consensus sequences TGGCAC and TTGCA/T, respectively (see Figure 5) (Merrick, 1993; Morett and Buck, 1989). In addition, σ^{54} family members also use a distinct pathway of open complex formation as compared to σ^{70} family members (Gruber and Gross, 2003). Whereas for all σ^{70} family members, the process of open complex formation is driven entirely by using free energy from binding and requires no coupled hydrolysis of nucleotides or other free energy inputs, open complex formation by the σ^{54} family requires nucleotide hydrolysis that is driven by interaction with a specialized bacterial enhancer-binding protein (EBP) (Popham et al., 1989; Rombel et al., 1998; Wedel and Kustu, 1995). EBPs belong to a large family of chaperone-like ATPases that are involved in the folding and remodeling of protein and protein/nucleic acid complexes (Studholme and Dixon, 2003). Bacteria use a variety of different types of EBPs to effect transcription from σ^{54}-dependent promoters (Studholme and Dixon, 2003). These transcriptional activators typically bind at unusually long distances from the promoter to mediate their effect, which is consistent with a DNA-looping mechanism of action. Thus, the interaction between the transcriptional activator (EBP) and the RNA polymerase occurs through DNA looping, which is a ubiquitous form of transcriptional control that allows interactions between DNA elements separated over long distances. The binding site for the transcriptional activator is usually found at 100 bp upstream from the transcriptional start site. However, these binding sites can function at much greater distances (up to 3 kb from the transcriptional start site) and have also been found downstream of the promoter region in some bacteria (Buck et al., 2000; Jyot et al., 2002; Studholme and Dixon, 2003; Xu and Hoover, 2001). DNA looping is required for the transcriptional activator to make physical contact with the σ54-holoenzyme that is bound as a stable closed complex to the –12 and –24 promoter elements. The result of this looping interaction is an activator-mediated nucleotide hydrolysis that converts the closed promoter complex to an open one ready to initiate transcription.

As mentioned above, the majority of alternative sigma factors are structurally and functionally related to the principal sigma factor in *E. coli*, σ^{70}, and are thus classified as belonging to the σ^{70} family. The σ^{70} family has been divided into four groups on the basis of phylogenetic relatedness (Lonetto et al., 1992, 1994). Group 1 contains σ^{70} and its homologs in other bacteria. Group 1 sigmas are the primary sigma factors that are responsible for the

majority of transcription in exponentially growing bacteria. Group 2 sigmas are closely related to the primary sigma factors but are not essential under standard physiologic growth conditions, i.e., they are dispensable for bacterial cell growth. Sigma S (RpoS), the general stress response regulator in many Gram-negative bacteria, is the most extensively studied member of this group. Group 3 σ factors are more distantly related to σ^{70} and can be further categorized into functionally related groups of proteins that activate regulons in response to a specific signal or developmental checkpoint, like heat shock, sporulation, and flagellar biosynthesis. The more divergent extracytoplasmic function (ECF) sigmas comprise group 4 sigmas. Most bacteria contain several members of the ECF sigma family that control a variety of functions in response to extracytoplasmic (i.e., extracellular) signals, like the presence of misfolded proteins in the bacterial periplasmic space, the presence of toxic molecules in the external environment, the presence of light, and changes in osmolarity or barometric pressure (Missiakas and Raina, 1998; Wosten, 1998). In addition, some have proposed the addition of a fifth family of σ^{70} family members, based on the recently described sigma factor TxeR of the nosocomial pathogen *Clostridium difficile* (which is distantly related to the ECF sigmas) and other positive regulators of toxin and bacteriocin genes (Helmann, 2002). While most bacteria have a single group 1 primary sigma factor, they have a variable number of other group sigma members, reflecting in part the different physiological and developmental characteristics of the organisms.

Protein sequence alignments of σ^{70} family members have identified four conserved regions of homology, each of which can be further divided into subregions (Figure 4) (Paget and Helmann, 2003). The function of these conserved regions has been studied by examining the effect of mutations in these regions on sigma interactions with core RNA polymerase, promoter recognition, strand melting, and transcriptional activators (Burgess and Anthony, 2001; Gruber and Gross, 2003; Paget and Helmann, 2003). Regions 2 and 4 are strongly conserved between all σ^{70} family members and contain subregions involved in binding to the core RNA polymerase and the –10 and –35 promoter regions (regions 2.4 and 4.2, respectively), and promoter melting (region 2.3). Region 4 has also been linked to the binding of transcriptional activators (Burgess and Anthony, 2001; Gruber and Gross, 2003; Kumar et al., 1994; Paget and Helmann, 2003). Much of region 1 is conserved only between the primary and closely related sigma factors (i.e., groups 1 and 2), and is thought to function as an autoinhibitory domain that masks the DNA-binding activity of free σ^{70}, thus preventing σ^{70} from binding to promoter sequences when it is not bound to RNA polymerase. Region 3 is weakly conserved among σ^{70} family members (e.g., it is virtually absent from ECF sigma factors). This region includes subregion 3.0 (formerly named 2.5), which interacts with DNA upstream of the –10 promoter sequence in some extended –10 promoters that lack the –35 element. The division of σ^{70} family members into functionally distinct regions based on amino acid sequence

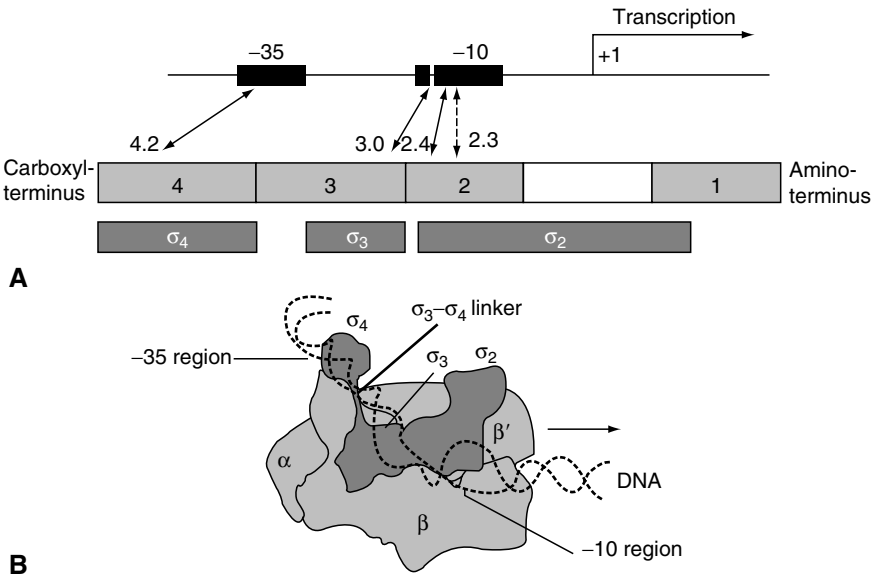

FIGURE 4. Structural characteristics of *Escherichia coli* σ^{70}. (A) The protein sequence has been divided into four regions on the basis of sequence conservation with other members of the σ^{70} family. Residues in the carboxy-terminal part of region 4 (subregion 4.2) form a helix-turn-helix motif that contacts the –35 element of the promoter. Residues from conserved regions 2 and 3 cooperate to mediate recognition of the –10 region and melting of the DNA. A residue in the amino-terminal part of region 3 (3.0) contacts the conserved TG motif in the extended –10 element of certain promoters that do not require a –35 region. Residues from an α helix in region 2 that correspond to the conserved subregions 2.3 and 2.4 interact intimately with the –10 element. Subregion 2.3 is thought to interact primarily with single-stranded DNA in the open complex (dashed arrow). The three domains of the sigma factor observed by X-ray crystallography (σ_2, σ_3, and σ_4) are indicated underneath the linear structure. Note that the protein domains correspond closely (although not precisely) with the regions assigned by sequence comparisons. (B) A model for the interaction of RNA polymerase holoenzyme (containing, β, β′, two α, and one ω subunit in addition to the sigma factor) with promoter DNA. The model is based on crystallographic analyses of σ domains, holoenzyme, and holoenzyme/model DNA complexes. The major functional domains of the sigma factor are shown in dark gray. The bold arrow indicates the direction of transcription. Although the template strand in the transcription bubble passes underneath the β unit and the σ_2 domain, the path of the DNA is shown throughout its length. (Adapted from Paget and Helmann, 2003.)

alignments has been verified recently by high-resolution structural data (Burgess and Anthony, 2001; Gruber and Gross, 2003; Paget and Helmann, 2003). Specifically, the structure of three stably folded domains of the housekeeping sigma factor from the bacteria *Thermus aquaticus* was solved using X-ray crystallography (Campbell et al., 2002). Combined with RNA

polymerase holoenzyme structures from *T. aquaticus* and the closely related *T. thermophilus*, these images have provided structural confirmation of the functional properties of σ, and we now have a fairly good picture of the bacterial transcription machinery containing the principal σ factor (Murakami et al., 2002b; Vassylyev et al., 2002).

As mentioned above, there is also a second family of bacterial sigma factors, the σ^{54} family, which is widely distributed in prokaryotes and is quite distinct both structurally and functionally from the σ^{70} family. Indeed, studies have demonstrated that the mode of transcription initiation mediated by σ^{54} may have more in common with that found in eukaryotes than that which occurs with σ^{70} and its prokaryotic relatives (Kornberg, 1998). While most bacteria contain multiple alternative sigma factors belonging to the σ^{70} class, they rarely have more than one belonging to the σ^{54} class (Buck et al., 2000).

It is important to keep in mind that all of these sigma factors operate together to effect proper regulation of cellular processes, i.e., they are not mutually exclusive. Moreover, sigma factors often control multiple regulatory pathways (including temporal regulation of different sigma factors within these pathways), thus adding to the complexity of transcriptional regulatory mechanisms in bacteria. In addition, many sigma factors are active at the same time, which presents the bacterial cell with a dilemma: how to determine which of them binds to the core RNA polymerase? Since sigma factors compete for binding to the same core RNA polymerase, the bacterial cell has evolved an impressive number of regulatory strategies to ensure that the appropriate sigma is associated with RNAP under any given condition. These strategies include mechanisms to regulate the amount and activity of every sigma in the cell as well as modifications to core RNA polymerase subunits (Gruber and Gross, 2003). In this regard, the use of antagonists to sigma factors, called antisigma factors, play an important role in controlling the activity of their respective sigmas in response to environmental cues (Hughes and Mathee, 1998).

Before engaging in more detailed discussion of alternative bacterial sigma factors, a word of caution—the nomenclature of sigmas can be confusing! In Gram-negative bacteria, the genes encoding sigma factors are designated *rpo* (for RNA polymerase subunit), whereas in Gram-positive bacteria, sigma genes are designate *sig* (for sigma factor). In addition, sigma factor proteins may be designated σ with a superscript letter (reflecting the gene name) or with a number (reflecting the molecular weight of the proteins). Originally, each sigma factor was designated with the Greek letter sigma (σ) with a superscript kilobase value representing the approximate mass of the protein (σ^{70}, σ^{38}, σ^{32}, etc.). Upon further investigation of reported protein weights and constant renaming of the sigma factors, they took on an alphabetically designated application (σ^{A}, σ^{S}, etc.). There is still division among researchers as to whether to base sigma nomenclature on molecular weights or letter designations.

2. Bacteria Use Alternative Sigma Factors to Regulate the Expression of Virulence Genes

Many pathogenic bacteria use alternative sigma factors for responding to environmental conditions that they encounter inside the infected host, including changes in nutrient availability, pH, osmolarity, and toxic oxidative products (Anonymous, 2000; Wick and Egli, 2004). Moreover, as shown in Table 1, bacterial pathogens often contain several alternative sigma factors (Mittenhuber, 2002). As discussed above, the association of alternative sigma factors with core RNA polymerase provides a mechanism for coordinately regulating the expression of entire families of genes (regulons) in response to diverse environmental conditions. In this regard, the use of global transcriptional (DNA microarrays) and proteomic (two-dimensional gel electrophoresis and mass spectrometry) techniques have been used to define the stress response stimulons in a variety of bacterial pathogens (Abshire and Neidhardt, 1993; Burns-Keliher et al., 1998; Cash, 2003; Cordwell et al., 2001; Hecker and Engelmann, 2000; Jungblut, 2001; Rosen and Ron, 2002; Schoolnik, 2002; VanBogelen et al., 1997, 1999). Given the wide variety of stressful microenvironments encountered by a bacterial pathogen during the infection process, it is not surprising that bacteria use alternate sigma factors to regulate the expression of genes encoding virulence factors (Hughes and Mathee, 1998). This chapter will review specific examples of several alternative sigma factors used by bacterial pathogens that have been implicated in the infection process, with focus on their regulation, function, and roles in mediating disease.

2.1. Sigma 38—The Major Stress Response Regulator

The alternative sigma factor σ^{38} is the master regulator of the general stress response in *E. coli* and *Salmonella*. Sigma 38, or RpoS (encoded by the *rpoS* gene) is important for the survival of various Gram-negative bacteria under extreme conditions, and controls the expression of a large number of genes that confer resistance to multiple stresses, including starvation, osmotic stress, acid shock, heat and cold shock, near-uv radiation, oxidative stress, and transition to stationary phase (Hengge-Aronis, 2000, 2002b). Not surprisingly, *rpoS* mutants are more susceptible than the wild type to these types of environmental stresses (Hengge-Aronis, 2000, 2002b). Because many of these stresses are relevant to those encountered in the host during the infection process, it is not surprising that numerous virulence genes in a variety of bacterial pathogens have been found to be regulated by σ^{38} (Hengge-Aronis, 2000; Yildiz and Schoolnik, 1998). First identified in *E. coli* for its central role in regulating the expression of genes important for cellular viability during stationary phase, the name σ^{S} or RpoS was proposed (Lange and Hengge-Aronis, 1991). Stationary phase presents the bacterial cell with a complex series of stresses, including conditions of nutrient deprivation, accumulation of toxic metabolites, oxidative injury, and alterations in pH. Accordingly,

TABLE 1. Alternative sigma factors of bacterial pathogens.[1]

Pathogen	Number of known factors	Number of ECF sigma factors	Reference (Adapted from Mittenhuber et al., 2002)
Bordetella pertussis	12	8	Parkhill et al., 2003
Borrelia burgdorferi	3	0	Fraser et al., 1997
Campylobacter jejuni	3	0	Parkhill et al., 2000
Escherichia coli K12	7	2	Blattner et al., 1997
Haemophilus influenzae	4	2	Fleischmann et al., 1995
Helicobacter pylori	3	0	Tomb et al., 1997
Legionella pneumophila	6	1	Cazalet et al., 2004
Listeria monocytogenes	4	1	Nelson et al., 2004
Mycobacterium leprae	4	2	Cole et al., 2001
M. tuberculosis	13	10	Cole et al., 1998
Mycoplasma pneumoniae	1	0	Himmelreich et al., 1996
Neisseria gonorrhoeae	3	1	Unpublished
N. meningitidis	4	1	Parkhill et al., 2000
Pseudomonas aeruginosa	24	19	Stover et al., 2000
Rickettsia prowazekii	2	0	Andersson et al., 1998
Salmonella typhimurium	7	2	McClelland et al., 2001
Staphylococcus aureus	3	0	Kuroda et al., 2001
Streptococcus pyogenes	3	0	Ferretti et al., 2001
Treponema pallidum	5	1	Fraser et al., 1998
Vibrio cholerae	8	3	Heidelberg et al., 2000
Ureaplasma urealyticum	1	0	Glass et al., 2000

[1] Adapted from Mittenhuber (2002).

researchers quickly discovered that σ^S was not just a regulator of stationary phase responsive genes but also controlled the expression of genes in response to a wide variety of stresses (Hengge-Aronis, 2000; Yildiz and Schoolnik, 1998). Controlling the expression of over 70 genes in *E. coli*, σ^{38} (σ^s, RpoS) is now more appropriately referred to as the master regulator of the general stress response, and has been found in a variety of enteric and related Gram-negative bacteria (Hengge-Aronis, 2000, 2002a). The adaptive stress response mediated by σ^{38} is particularly important for growth-restricted cells. Remarkably, σ^{38} confers bacterial resistance not only to the stress encountered but also to a variety of stresses that they have not yet encountered (referred to as "cross-protection"). This adaptive response is particularly important for cells in a growth-restricted state, and serves to equip them with multiple stress resistance in preparation for surviving future stresses. This multiple stress resistance is in stark contrast to that mediated by specific stress responses (as we will see later in this chapter), which are activated by a single stress signal and control the expression of proteins that provide protection against that specific stress only. While specific stress responses act to eliminate the offending stress and/or repair cellular damage that has already occurred, the general stress response renders cells broadly resistant such that damage is avoided rather than requiring repair. Thus, by providing

cross-protection against multiple stresses, the major function of the general stress response is preventative in nature. (Hengge-Aronis, 2002b).

2.1.1. Sigma 38 Is Closely Related to the Major Housekeeping Sigma Factor, σ^{70}

Sigma 38 and σ^{70} are structurally and functionally very similar and recognize similar promoter sequences (Hengge-Aronis, 2002c). Indeed, these two sigma factors use the same basic core promoter elements in the –10 and –35 regions; however, they control different sets of genes in vivo (Hengge-Aronis, 2002c). Until fairly recently, the basis for promoter selection between RNA polymerase carrying σ^{38} or σ^{70} was puzzling, as it appeared to contradict the rationale of promoter recognition by different sigma subunits of RNA polymerase. It now appears that a combination of distinct sequence and structural features of a promoter play an important role in determining σ^{38} promoter specificity (Hengge-Aronis, 2002c). These features include: (1) sequence elements surrounding the core –10 and –35 regions (i.e., a degenerate –35 sequence, A-T rich regions downstream of the –10 sequence, a distal half-site of an UP element, and a cytosine at the –13 position); (2) deviations from the optimum spacer length between the –10 and –35 regions; (3) local DNA topology; and (4) *trans*-acting regulatory factors (Figure 5) (Hengge-Aronis, 2002c). In addition, many σ^{38}-controlled promoters contain multiple binding sites for additional transcriptional regulators such as cAMP-CRP, H-NS, Lrp, IHF, and Fis (Hengge-Aronis, 2002c). It is important to note that these factors are modular by design, with different combinations of these features being used to generate σ^{38} promoter specificity (Hengge-Aronis, 2002c). In addition to "fine-tuning" promoter selectivity, bacteria also use other mechanisms to enhance σ^{38} activity relative to that of σ^{70} (Hengge-Aronis, 2000,

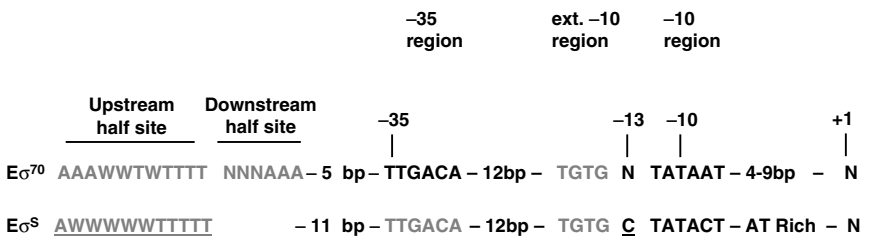
–35 region
ext. –10 region
–10 region
Upstream half site
Downstream half site
–35
–13
–10
+1
Eσ70 AAAWWTWTTTT NNNAAA – 5 bp – TTGACA – 12bp – TGTG N TATAAT – 4-9bp – N
EσS AWWWWWTTTTT – 11 bp – TTGACA – 12bp – TGTG C TATACT – AT Rich – N

FIGURE 5. Comparisons of consensus sequences of promoters preferentially activated by RNA polymerase holoenzyme containing σ^{70} (upper line) and σ^{38} (lower line). Elements highlighted in gray are optional (i.e., present only in a minority of the respective group of promoters). In general, the –35 region in σ^{38}-dependent promoters tends to be more degenerate than in σ^{70}-controlled promoters. Underlined elements contribute to σ^{S} selectivity of a promoter (see text for details). ext, extended. (Adapted from Hengge-Aronis, 2002c.)

2002b, c; Jishage et al., 2001). These mechanisms include the use of: (1) the anti-σ^{70} factor, Rsd, which binds to σ^{70} and prevents its interaction with RNA polymerase during stationary phase; (2) the small "alarmone" molecule ppGpp (whose cellular levels rise in response to amino acid limitation); and (3) 6S RNA, which accumulates to high levels in the bacterial cell during stationary phase and binds to RNA polymerase holoenzyme containing σ^{70}, thereby diminishing its transcriptional ability (Hengge-Aronis, 2002c).

2.1.2. The Regulation of σ^{38} Is Complex

Regulation of σ^{38} occurs at multiple levels, including transcription, posttranscription, translation, and protein stability. To add to this complexity, various levels of σ^{38} regulation are differentially affected by various stress conditions (Figure 6) (Hengge-Aronis, 2002c). This multilevel control of σ^{38} synthesis and accumulation/stability provides the potential for "fine-tuning" the integration of multiple signals, which result in distinct and complex regulatory patterns (Hengge-Aronis, 2002b). While σ^{38} regulation occurs at multiple levels, the majority of regulation in *E. coli* (and *Salmonella*) occurs at the translational and posttranslational levels, with different stresses acting independently at different steps (Hengge-Aronis, 2002b). Interestingly, *Pseudomonas aeruginosa* appears to regulate *rpoS* more at the transcriptional level than do either *E. coli* or *Salmonella* (Venturi, 2003). However, since the vast majority of studies on σ^{38} regulation have been done in *E. coli*, we shall use it as the model for our discussion of this topic.

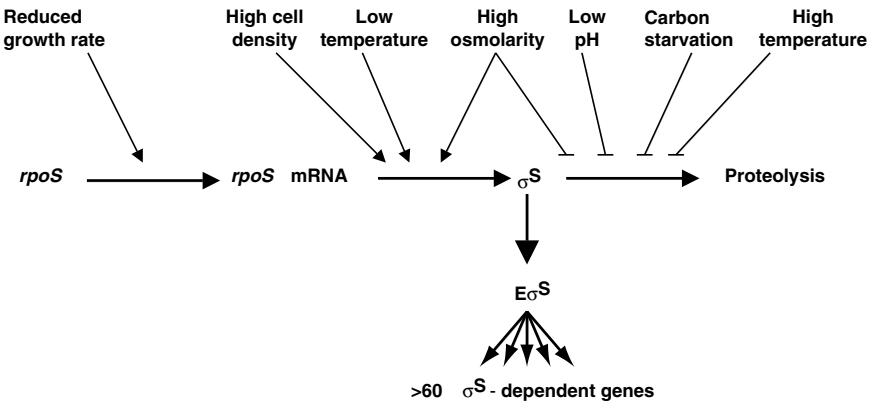

FIGURE 6. Various levels of σ^S (σ^{38}, RpoS) regulation are differentially affected by various stress conditions. An increase in the cellular σ^S level can be obtained either by stimulating σ^S synthesis at the levels of *rpoS* transcription or *rpoS* mRNA translation or by inhibiting σ^S proteolysis (which under nonstress conditions is extraordinarily rapid). (Adapted from Hengge-Aronis, 2002b, and reprinted with permission from the American Society for Microbiology, Bacterial Stress Responses, Storz and Hengge-Aronis, Editors, 2000, ASM Press, Washington, D.C.).

Increased levels of RpoS protein are found in bacteria encountering nutrient deprivation or other stress conditions that lead to a cessation or slowing of cell growth (Hengge-Aronis, 2002b). Rapidly growing bacterial cells (i.e., in exponential phase) contain low levels of σ^{38} protein due to rapid degradation by proteolysis (Hengge-Aronis, 2000, 2002b). However, σ^{38} protein stability increases dramatically when cells are subjected to relatively threatening conditions, like carbon starvation (Lange and Hengge-Aronis, 1994; Takayanagi et al., 1994), osmotic upshift (Muffler et al., 1996c), acidic pH (Bearson et al., 1996), and high temperature (Muffler et al., 1997). The increased stability of σ^{S} protein in response to environmental stress makes sense, as it provides a protective mechanism to ensure cell viability. The basic translational control of *rpoS* uses *rpoS* mRNA secondary structure and *trans*-acting factors that bind to this structure. Under normal physiological conditions, *rpoS* mRNA contains a secondary structure that blocks access of the ribosomes to the Shine–Dalgarno sequence and thus impedes efficient translation (Hengge-Aronis, 2002b).

However, in response to different environmental stresses, translation of *rpoS* is thought to be stimulated by rearrangements of *rpoS* mRNA secondary structure that render the ribosomal binding site accessible to the protein-synthesizing machinery. In this regard, several proteins (Hfq, HU, and HNS) and small regulatory RNAs (DsrA, RprA, and OxyS) regulate translation by affecting the secondary structure of *rpoS* mRNA (Balandina et al., 2001; Barth et al., 1995; Brown and Elliott, 1996; Lease and Belfort, 2000; Lease et al., 1998; Majdalani et al., 1998, 2001; Muffler et al., 1996b; Sledjeski et al., 1996; Yamashino et al., 1995; Zhang et al., 1998). DsrA and RprA function to promote *rpoS* translation, while OxyS inhibits translation. Interestingly, *rpoS* is the only known gene regulated posttranscriptionally by at least three small regulatory RNAs (Hengge-Aronis, 2002b). Cellular levels of RpoS are further regulated by posttranslational alterations in protein stability through the action of proteases. The molecular components regulating RpoS stability include the ClpXP protease and the response regulator protein RssB (SpreE), which is required for targeting σ^{38} to the ClpXP protease for degradation (Andersson et al., 1999; Bearson et al., 1996; Becker et al., 1999; Muffler et al., 1996a; Pratt and Silhavy, 1996; Schweder et al., 1996; Zhou et al., 2001). Regulation of the phosphorylation state of RssB is essential in determining the turnover rate (and hence stability) of σ^{38} (Becker et al., 2000; Bouche et al., 1998).

Specifically, under normal physiological growth conditions (i.e., non-stress), RssB exists in a phosphorylated form, which has a high affinity for binding σ^{38}. Binding of RssB to σ^{38} facilitates transfer of σ^{38} to ClpXP, and its subsequent degradation. This is why levels of σ^{38} are low in exponentially growing cells. However, during times of stress, RssB is believed to exist in a dephosphorylated state, in which it has reduced affinity for binding σ^{38}, thus increasing σ^{38} stability (Figure 7). While a thorough discussion of σ^{38} regulation is beyond the scope of this chapter, the reader is referred to several excellent reviews in this area (Hengge-Aronis, 2000, 2002c; Wick and Egli, 2004).

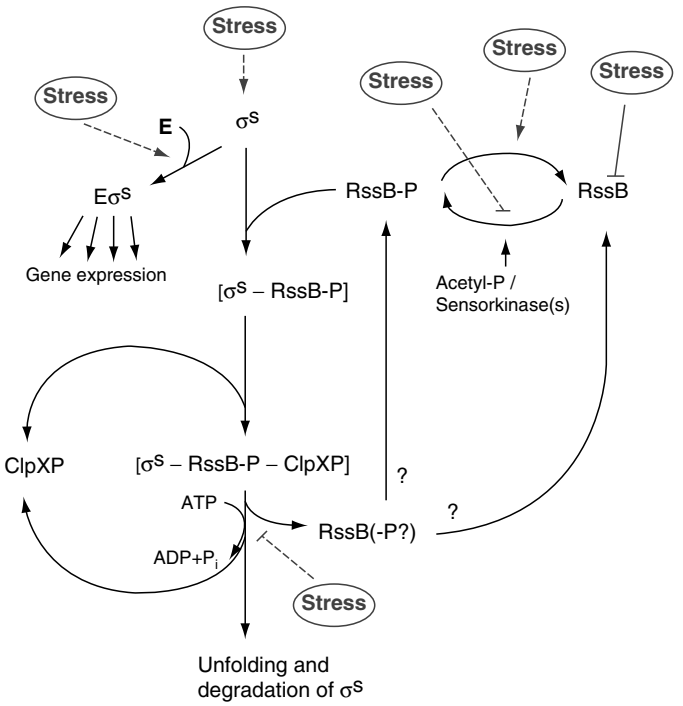

FIGURE 7. Role of RssB/ClpXP and putative signal input in the σ^S recognition and degradation pathway. The response regulator RssB is an essential, specific, and direct σ^S recognition factor. RssB delivers σ^S to the ClpXP protease, where σ^S is unfolded and completely degraded, whereas RssB is released. Sigma S binding requires RssB phosphorylation, but it is unclear whether the catalytic cycle of RssB involves obligatory dephosphorylation during release and subsequent rephosphorylation. Stress signals may affect (1) the phosphorylation of RssB and therefore RssB-S complex formation; (2) the cellular level of RssB (which in growing cells is rate-limiting for σ^S proteolysis); (3) the synthesis of σ^S such that RssB becomes titrated on σ^S overproduction; (4) σ^S association with RNA polymerase core enzyme, which protects against binding by RssB; and (5) the function of the ClpXP protease itself (see text for details). However, the molecular details of the stress signal input pathways involved are still largely unknown. (Adapted from Hengge-Aronis, 2002b and reprinted with permission from the American Society for Microbiology, Bacterial Stress Responses, Storz and Hengge-Aronis, Editors, 2000, ASM Press, Washington, D.C.).

2.1.3. Sigma 38 Regulates Bacterial Virulence in a Variety of Gram-negative Organisms

While most work on the RpoS regulon has been in *E. coli*, homologs of σ^{38} are found in a number of Gram-negative bacterial pathogens, including foodborne pathogens like *Salmonella* spp., *Shigella* spp., *Yersinia* spp., and *Vibrio vulnificus*; respiratory pathogens like *P. aeruginosa*, *Burkholderia pseudoma-*

llei, and *Legionella pneumophila*; the spirochete *Borrelia burgdorferi*, the causative agent of Lyme disease; and the plant pathogenic *Erwinia* spp. and *Ralstonia solanacearum* (Aguilar et al., 2003; Andersson et al., 1999; Flavier et al., 1998; Hales and Shuman, 1999; Hulsmann et al., 2003; Martinez-Garcia et al., 2001; Mukherjee et al., 1998; Park et al., 2004; Small et al., 1994; Subsin et al., 2003). Moreover, RpoS has been shown to regulate stress response and/or virulence genes in several of these pathogens, and is important for their disease-causing potential, e.g., *Salmonella* (Fang et al., 1992), *Pseudomonas* (Jorgensen et al., 1999), *Legionella* (Hales and Shuman, 1999), *Erwinia* (Andersson et al., 1999), *B. burgdorferi* (Caimano et al., 2004), and *Shigella flexneri* (Small et al., 1994) (discussed below). The role of RpoS in bacterial pathogenesis has been best characterized in the model facultative intracellular pathogen, *S. typhimurium*.

In *S. typhimurium*, an *rpoS* mutant is susceptible to a variety of stresses that are relevant to those encountered during infection, including nutrient deprivation, oxidative stress, osmotic stress, acid stress, and DNA damage (Fang et al., 1992). Indeed, the activity of RpoS and RpoS-regulated genes has been shown to increase after *S. typhimurium* entry into both macrophages and epithelial cells (Chen et al., 1996). While the nature of the signal(s) responsible for *Salmonella* RpoS induction within mammalian host cells is unknown, it might be related to the slow growth rate of the bacteria within the host cell membrane-bound vacuole, where the organisms encounter nutrient restriction and other stresses (Chen et al., 1996). In mice, RpoS is required for the ability of *S. typhimurium* to cause a lethal systemic infection (Fang et al., 1992). Indeed, the lethal dose 50 (LD50) of an *rpoS* mutant of *S. typhimurium* is at least 1,000-fold higher than that of the wild-type parent strain when administered to mice via the oral route of infection (Fang et al., 1992; Nickerson and Curtiss, 1997). Interestingly, σ^{38}-overproducing *Salmonella* strains also exhibit reduced virulence in mice (Coynault et al., 1996). This finding underscores an important point regarding production of bacterial virulence factors—namely that their expression levels are critical and are carefully controlled by the cell—and deviations from normal expression levels can profoundly impact the outcome of infection.

In *S. typhimurium* (and other *Salmonella* serovars) RpoS regulates the expression of the *Salmonella* plasmid virulence (*spv*) genes, which are necessary for the ability of orally administered *Salmonella* to effectively establish a systemic infection beyond Peyer's patches (Fang et al., 1992; Kowarz et al., 1994; Norel et al., 1992). The *spv* genes are involved in systemic infection by increasing the replication rate of the bacteria in host tissues beyond the intestines (Gulig and Doyle, 1993). Accordingly, *Salmonella rpoS* mutants are significantly impaired in their ability to colonize the spleens and livers of infected mice (Coynault et al., 1996; Nickerson and Curtiss, 1997; Wilmes-Riesenberg et al., 1997). In addition, *rpoS* mutations reduce the ability of *S. typhimurium* to colonize Peyer's patches of infected mice following oral infection (Coynault et al., 1996; Nickerson and Curtiss, 1997) and decrease

the persistence of virulence plasmid-cured strains in the spleen (Kowarz et al., 1994). These latter effects presumably result from the inappropriate expression of one or more unidentified *rpoS*-regulated chromosomal genes. RpoS also regulates the expression of thin aggregative fimbriae (also called curli fimbriae) in *Salmonella*, which mediate adhesion to intestinal epithelial cells and may contribute to virulence in mice (Dibb-Fuller et al., 1999; Sukupolvi et al., 1997; van der Velden et al., 1998). The human-restricted *Salmonella* serovars such as Typhi (which causes typhoid fever) have no virulence plasmid, and the role of *rpoS* in the virulence of these serovars is not as well understood. However, it is thought that RpoS-controlled *S. typhi* chromosomal genes may be important for the virulence of this pathogen. Specifically, an *rpoS* mutant of serovar Typhi is sensitive to acid, oxidative and starvation stress and is less cytotoxic for macrophages than the parental strain (Khan et al., 1998). Moreover, RpoS (along with RpoN/σ^{54}) controls the expression of the *S. typhi* O-antigen lipopolysaccharide (LPS), which is an important virulence factor in this organism (Bittner et al., 2004).

Collectively, these findings suggest that *rpoS* may be involved in the virulence of serovar Typhi in humans. In support of this suggestion, the attenuated *S. typhi* vaccine strain Ty21a, which is used as a live oral vaccine to prevent typhoid fever, is an *rpoS* mutant and is susceptible to a variety of environmental stresses. This suggests that the *rpoS* mutation may contribute to the safety of this strain in humans (Robbe-Saule et al., 1995). In addition, introduction of a wild-type *rpoS* gene into the live *S. typhi* oral vaccine strainTy21a enhanced the ability of the bacteria to survive starvation conditions and a variety of other stresses (Robbe-Saule et al., 1995).

Studies with other bacterial pathogens have also shown a role for RpoS in virulence. For example, in the enteric pathogen, *S. flexneri*, an *rpoS* mutant was shown to be more sensitive to acid stress and significantly less infective than the wild-type parental strain (Small et al., 1994). In the invasive enteropathogen, *Yersinia enterocolitica*, RpoS has been shown to affect cell survival following diverse stresses, such as high-temperature, oxidative, osmotic, and acid stress in a temperature-dependent manner (Badger and Miller, 1995). However, in this same study, the *Yersinia rpoS* mutant was not affected in expression of the virulence genes encoding the invasion proteins, *inv* and *ail*, invasion of tissue culture cells, or virulence in mice following growth at either temperature. In a separate report, RpoS was required for stationary phase expression of the heat-stable *Yersinia* enterotoxin (Yst) (Iriarte et al., 1995).

P. aeruginosa rpoS mutants show an increased susceptibility to a variety of stresses, including carbon starvation, heat, osmotic, oxidative, and acid stresses, although the survival defects observed for this pathogen are not as extreme as those for *E. coli* (Jorgensen et al., 1999; Suh et al., 1999). In addition, RpoS affects the production of several virulence factors in *P. aeruginosa*, including pyocyanin and exotoxin A, and has been implicated in quorum sensing and biofilm formation, as well as resistance to antibiotics (Schuster

et al., 2004; Suh et al., 1999; Whiteley et al., 2001). Recently, microarray analysis was used to identify the RpoS regulon in *P. aeruginosa* (Schuster et al., 2004). This study identified 772 genes (approximately 15% of the *P. aeruginosa* genome) that were differentially regulated by RpoS in the stationary phase of growth, many of which were important for biofilm formation. While several of the *P. aeruginosa* RpoS-controlled genes encoded similar functions to those regulated by RpoS in *E. coli*, many of the genes identified by microarray analysis encoded functions that were clearly distinct from those in *E. coli* and are not obviously related to a stress response. The authors of this study hypothesized that this finding may explain why *P. aeruginosa rpoS* mutants are not as sensitive to environmental stresses as the corresponding *E. coli* strains. In *V. cholerae*, RpoS is required for resistance to oxidative, osmotic, and nutrient deprivation stresses, as well as colonization of the mouse small intestine (Merrell et al., 2000; Yildiz and Schoolnik, 1998). In the intracellular pathogen, *L. pneumophila* (the causative agent of Legionnaire's disease) RpoS is required for growth and survival within its environmental protozoan host, *Acanthamoeba castellanie*; however, it is not required for stationary phase stress resistance (Hales and Shuman, 1999). Likewise, in the Lyme disease spirochete, *B. burgdorferi*, RpoS also does not appear to be important for the general stress response in vitro, since strains lacking RpoS survived a variety of stresses as well as the wild type (with the exception of acid stress). However, RpoS was important for the virulence of this pathogen in mice (Caimano et al., 2004), indicating the role of this sigma factor in bacterial adaptation during the infection process. Interestingly, it has been shown that regulation of the antigenic lipoproteins OspC and DbpA in *B. burgdorferi* is controlled by both RpoS and RpoN. In this regulatory pathway, RpoN controls the expression of RpoS, which in turn governs the expression of these lipoproteins (Hubner et al., 2001).

2.2. *Sigma B—The General Stress Response Regulator in Gram-positive Bacteria*

Just as σ^{38} regulates the general stress response in a variety of Gram-negative organisms, σ^{B} performs a similar function in many Gram-positive bacteria (Price, 2000). While Gram-negative bacteria recruit different sigma factors to the cytoplasmic and extracytoplasmic spaces to prevent damage to these compartments caused by stress (discussed later in this chapter), Gram-positive organisms contain only a single membrane-bound compartment and use cytoplasmic sigma factors for stress protection. Sigma B regulates the expression of more than 150 general stress response proteins that provide Gram-positive bacteria with nonspecific, preventive stress resistance (Hecker and Volker, 2001). In agreement with its central role in the general stress response, σ^{B} mutants are more sensitive to a variety of stresses, including severe heat and salt shocks, ethanol treatment, low or high pH, and free oxygen radicals (Antelmann et al., 1996; Engelmann and Hecker, 1996; Gaidenko and Price,

1998; Volker et al., 1999). The activity of σ^B is regulated by a signal transduction network in which key protein interactions are regulated by serine and threonine phosphorylation. In this section, we discuss some of the central features of σ^B regulation using the bacterium *B. subtilis* as the model organism. The role of σ^B in the virulence of the model Gram-positive pathogens *Staphylococcus aureus* and *Listeria monocytogenes* is also discussed.

Sigma B was the first alternative sigma factor discovered in bacteria (Haldenwang and Losick, 1979). First identified by Losick and Haldenwang as a factor involved in the sporulation of *B. subtilis*, it is now known that σ^B also controls a stress/starvation regulon comprising a large set of general stress genes (over 150), whose expression is induced when *B. subtilis* reaches stationary phase (Haldenwang, 1995; Haldenwang and Losick, 1979; Volker et al., 1994, 1999). These σ^B-dependent genes are induced in response to a variety of stresses, including heat and cold shock, ethanol, acid or salt stress, as well as by starvation for glucose, phosphate, and oxygen (Haldenwang, 1995; Hecker et al., 1996; Price, 2000). Just as for σ^{38}, the adaptive stress response mediated by σ^B is particularly important for the survival of cells in a growth-restricted state, and serves to equip them with multiple stress resistance to future stresses. The majority of genes central to the regulation and function of σ^B are encoded along with the structural gene for σ^B within the *sigB* operon (Figure 8) (Price, 2000). This operon encodes the following regulators of σ^B: RsbR, RsbS, RsbT, RsbU, RsbV, RsbW, and RsbX (where Rsb stands for regulator of sigma B) (Kalman et al., 1990; Wise and Price, 1995). Basal transcription expression levels of these genes during normal growth is driven from a σ^A (housekeeping sigma)-dependent promoter, which is located upstream of the first gene in the regulon (Kalman et al., 1990). An internal σ^B-dependent promoter drives expression of the genes *rsbV*, *rsbW*, *sigB*, and *rsbX* when σ^B is activated (Wise and Price, 1995).

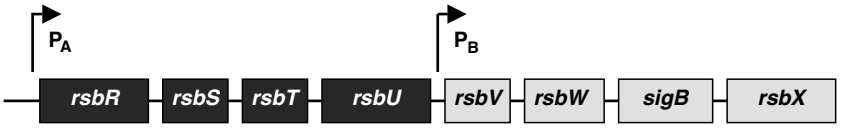

FIGURE 8. The structural gene for σ^B (*sigB*) is encoded within an eight-gene operon. The other genes encode products that posttranslationally regulate σ^B activity. The first four genes (*rsbR*, *rsbs*, *rsbT*, and *rsbU)* encode products that comprise the environmental signaling branch of the pathway that communicates with the common regulators encoded by three downstream genes (*rsbV*, *rsbW*, and *sigB*). The gene *rsbX* encodes a phosphatase (RsbX), the activity of which is thought to provide an indirect measure of σ^B levels in the cell. Expression of the *sigB* operon is under the control of two promoters (P): a σ^A-dependent promoter (P_A) and a σ^B dependent promoter (P_B). The genes encoding the antisigma factor (RsbW) and σ^B are under the control of the P_B promoter. (Adapted from Price, 2000.)

Activation of σ^B by the Rsb proteins in *B. subtilis* occurs through a complex phosphorylation/dephosphorylation cascade in response to various stresses that have been grouped into two broad types: environmental and metabolic. In *B. subtilis*, these two classes of stress signals are conveyed to σ^B through two interconnected but separate signaling pathways (Kim et al., 2004; Price, 2000). The environmental stress-signaling pathway is transmitted to σ^B by regulatory proteins encoded within the *sigB* operon. The "metabolic" or energy stress-signaling pathway is transmitted to σ^B by proteins encoded in a two-gene operon (*rsbQ*/*rsbP*) that is physically separate from the *sigB* operon. While both of these signaling pathways use separate phosphatases to communicate their respective stress information to σ^B, these phosphatases act on the same central regulator of σ^B, RsbV (Figure 9). Since the key elements of the σ^B regulatory network are largely encoded within the *sigB* operon, only the environmental stress-signaling pathway will be discussed in detail in this section.

RsbW is an antisigma factor that negatively regulates the activity of σ^B (Benson and Haldenwang, 1992). RsbV is a positive regulator of σ^B activity, and must bind to RsbW in order to exert its positive effect (Benson and Haldenwang, 1992; Boylan et al., 1993). The control of σ^B activation by these two proteins is dependent upon the phosphorylation state of RsbV, an anti-antisigma factor that regulates the action of RsbW by altering its ability to bind to either RsbV or σ^B (Alper et al., 1996; Benson and Haldenwang, 1993; Dufour and Haldenwang, 1994; Yang et al., 1996). During exponential growth, when increased levels of stress response proteins are not needed, the anti-antisigma factor RsbV is phosphorylated and cannot bind to the RsbW antisigma factor, which is then free to bind to σ^B and prevent it from interacting with core RNA polymerase. However, during conditions of growth restriction (i.e., stress), levels of nonphosphorylated RsbV increase due to the actions of two phosphatases: RsbU (environmental stress) and RsbP (energy stress). The nonphosphorylated form of RsbV binds to RsbW, which causes σ^B to be released from its inhibitory complex with RsbW. Once released, the activated σ^B is then free to bind with core RNA polymerase to induce expression of the *sigB* operon from the σ^B-dependent promoter. This results in increased levels of σ^B and the induction of the general stress response proteins. Thus, this phosphorylation regulatory mechanism serves to suppress the expression of the *sigB* regulon under nonstress conditions and allows rapid induction of the whole regulon in response to stress. In addition, recent studies on the induction of the *sigB* regulon have indicated that *sigB* can also perceive signals independent of the RsbV-mediated signal transduction pathways under certain stress conditions (Kim et al., 2004). While a comprehensive coverage of the complex genetic and biochemical regulation of σ^B is beyond the scope of this chapter, there are excellent reviews available on this topic (Kim et al., 2004; Price, 2000).

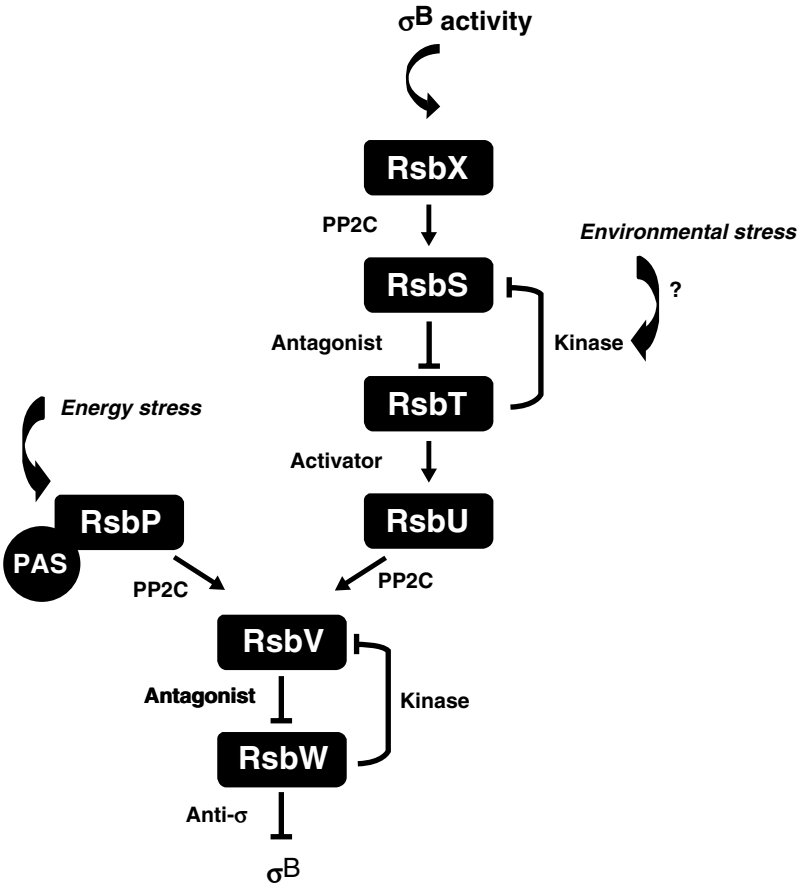

FIGURE 9. Model of σ^B regulation with two signaling pathways converging on RsbV-P, the antagonist form found in unstressed cells. The energy-stress signaling pathway terminates with the RsbP phosphatase, which contains an amino terminal region, which senses the overall energy level of the cell and communicates this information to its phosphatase domain. In contrast, the environmental-stress signaling pathway terminates with the RsbU phosphatase, which is activated by interaction with the RsbT protein. Whether RsbT binds and activates RsbU is regulated by upstream componenets. When activated by its particular class of stress, either the RsbP or the RsbU phosphatase removes the serine phosphate from RsbV-P. Dephosphorylated RsbV then binds the RsbW antisigma factor, which releases σ^B to activate its target general stress genes. The RsbX phosphatase provides a feedback loop that responds to increasing σ^B levels and prevents continued signaling. (Adapted from Price, 2000, and reprinted with permission from the American Society for Microbiology, Bacterial Stress Responses, Storz and Hengge-Aronis, Editors, 2000, ASM Press, Washington, D.C.).

2.2.1. Sigma B Regulates Bacterial Virulence in a Variety of Gram-positive Organisms

Multiple lines of evidence indicate a broad role for σ^B and σ^B-regulated genes in the virulence of Gram-positive bacteria. For example, σ^B has been shown to regulate the stress resistance and virulence of *L. monocytogenes*, a facultative intracellular foodborne pathogen that is associated with serious invasive infections in susceptible humans and animals. Numerous studies have shown that *L. monocytogenes* strains carrying a mutant *sigB* gene are sensitive to a variety of stresses that are relevant to those encountered by the pathogen (1) during infection of the intestinal tract, including osmotic, oxidative, and acid stresses (Chaturongakul and Boor, 2004; Ferreira et al., 2001, 2003; Wiedmann et al., 1998); and (2) during food processing, including high hydrostatic pressure and freeze survival (Wemekamp-Kamphuis et al., 2004). In addition, σ^B has been shown to regulate the expression of several *L. monocytogenes* virulence genes, including those encoding the central virulence gene regulator PrfA, the bile salt hydrolase Bsh (which is important for bacterial survival of bile salt detergents in the intestinal tract), the internalin genes *inlA* and *inlB*, which are important for invasion of mammalian cells, and the stress response gene *opuCA* (Nadon et al., 2002; Sue et al., 2003, 2004).

In agreement with these findings, studies have demonstrated that σ^B contributes to *L. monocytogenes* invasion of human intestinal epithelial cells and resistance to bile salts (Begley et al., 2005; Kim et al., 2004). Moreover, animal infection studies have also indicated that σ^B is important for *L. monocytogenes* virulence (Kazmierczak et al., 2003; Nadon et al., 2002; Wiedmann et al., 1998). For example, a *L. monocytogenes* σ^B mutant strain demonstrated a reduced ability to colonize the liver and spleen in mice following infection as compared to the wild-type parent strain (Nadon et al., 2002). Other animal studies using *L. monocytogenes* strains carrying mutations in various σ^B-regulated genes have also implicated the involvement of σ^B in the virulence of this pathogen (Begley et al., 2005; Nadon et al., 2002). In addition, σ^B also appears to play a role in the virulence of *S. aureus*, and has been linked with regulation of virulence and stress gene expression in this organism (Bischoff et al., 2001; Deora et al., 1997; Gertz et al., 2000; Manna et al., 1998). As for *L. monocytogenes*, *S. aureus* σ^B mutant strains show enhanced sensitivity to a variety of stresses relevant to those encountered during the infection process as compared with wild-type cells (Chan et al., 1998; Kullik et al., 1998). The involvement of σ^B in the pathogenesis of *S. aureus* was suggested when it was found that σ^B contributes to the transcriptional regulation of the gene encoding the DNA-binding protein SarA (Cheung et al., 1999; Deora et al., 1997; Gertz et al., 2000; Manna et al., 1998). Sar A modulates the expression of the global virulence regulator gene, *agr*, that controls the synthesis of numerous extracellular and cell surface proteins involved in the pathogenesis of *S. aureus* (Chien and Cheung, 1998; Chien et al., 1998; Morfeldt et al., 1996). In addition, σ^B has been shown to be important for the ability of *S. aureus* to

cause arthritis and sepsis in a mouse model of infection, and to persist in the kidneys and joints of these animals (Jonsson et al., 2004).

Sigma B has also been shown to influence the internalization of *S. aureus* into osteoblasts (bone-forming cells), and could contribute to strain-dependent differences in the ability of this pathogen to cause infections of the bone (Nair et al., 2003). Recently, σ^B was shown to regulate biofilm formation in *S. epidermidis,* a leading cause of foreign body–associated infections and a frequent nosocomial pathogen (Knobloch et al., 2004). This organism has an inherent capacity to adhere to catheters and other indwelling devices and form a multicellular community, known as a biofilm, which is then difficult to combat with host defenses or antibiotics. Sigma B has also been shown to contribute to the virulence of *B. anthracis*, the causative agent of anthrax and of obvious concern as a potential agent of bioterrorism (Fouet et al., 2000).

In addition to the general stress response sigmas (σ^{38} and σ^B) that provide general and rather nonspecific protection under a variety of adverse conditions, bacteria also use stress-specific sigma factors to induce a set of specific stress proteins that confer protection against a particular stress. These sigma factors are discussed below.

2.3. Sigma 32—Heat Shock

In response to a sudden increase in temperature (called heat shock), bacteria induce the expression of a specific set of genes whose protein products (called heat shock proteins or HSPs) are highly conserved and designed to promote survival of the cell at elevated temperatures (Guisbert et al., 2004; Rosen and Ron, 2002; Yura et al., 2000). The main strategy of the bacterial heat shock response is defense from denatured (i.e., unfolded and misfolded) proteins, which accumulate during thermal stress. Accordingly, the bacterial HSPs are designed to repair damage to proteins caused by thermal stress. Many bacterial HSPs are molecular chaperones or proteases that function by facilitating refolding of damaged proteins or degrading proteins that cannot be repaired (Guisbert et al., 2004; Yura et al., 2000). This adaptive response is essential for cell survival during thermal stress, since denatured proteins are unable to perform their specific cellular functions. Since cellular survival depends on maintaining proteins in their proper functional conformations, the cell carefully regulates the expression of chaperones and proteases to compensate for heat shock, as well as other stresses that cause abnormal protein folding; including exposure to ethanol, heavy metals, DNA-damaging agents, antibiotics, oxidative stress, and phage infection (Guisbert et al., 2004; Yura et al., 2000). The bacterial heat shock response has been studied most extensively in *E. coli*, which have two different heat shock sigma factors: one that serves the cytoplasmic compartment (σ^{32}) and one that serves the extracytoplasmic compartment (i.e., cell envelope) σ^E. Both heat shock sigmas are induced by excessive unfolded proteins that result from heat shock and other stresses and provide major protection against protein damage (in the cytoplasm for σ^{32},

and in the periplasm for σ^E). Moreover, the heat shock regulons controlled by these two sigma factors are interrelated at very high temperatures (45–50˚C), when σ^E uniquely regulates the expression of the σ^{32} heat shock regulon (Guisbert et al., 2004; Silhavy, 2000). In this section, we discuss the cytoplasmic heat shock response mediated by σ^{32}. The role of σ^E in sensing and responding to misfolded proteins in the cell envelope is described later in this chapter.

Originally discovered in *E. coli*, σ^{32} (encoded by the *rpoH* gene) is responsible for the transcription of genes whose proteins promote cellular survival at elevated temperatures, and in response to other stresses (Arsene et al., 2000; Grossman et al., 1984; Guisbert et al., 2004; Herman, 2000; Landick et al., 1984; Yura et al., 1984, 2000). Sigma 32 is responsible for the expression of over 30 known HSPs that specifically protect the cell against cytoplasmic damage caused by protein misfolding (Arsene et al., 2000; Herman, 2000; Yura et al., 2000). As mentioned above, many of these HSPs are molecular chaperones (like GroEL-GroES, DnaK-DnaJ, and GrpE) or ATP-dependent proteases (like Clp and FtsH) that help in the folding and repair of cytoplasmic proteins during heat stress (Arsene et al., 2000; Guisbert et al., 2004; Herman, 2000; Yura et al., 2000). The pertinent question we must ask concerning heat shock regulators is how the activity of these proteins is modulated after a heat shock. Studies with *E. coli* have shown that intracellular levels of σ^{32} (and thus HSPs) are low during growth at 30˚C, but rapidly increase following a temperature shift to 42˚C (referred to as the induction phase of the heat shock response) (Arsene et al., 2000; Guisbert et al., 2004; Herman, 2000; Yura et al., 2000). The induction phase is transient, lasting only for several minutes, and σ^{32} (and HSP) levels gradually decline during the adaptation phase to achieve a new steady-state level within 20–30 min, which is slightly higher than the preshift level (Straus et al., 1987). While control of intracellular σ^{32} levels occurs at multiple levels, two posttranscriptional regulatory mechanisms are key in this process: (1) regulation of the synthesis of σ^{32} by translation of the *rpoH* gene (mediated by a temperature-directed signaling pathway); and (2) activity and stability of the σ^{32} protein (mediated by the chaperone- and protease-mediated pathway) (Arsene et al., 2000; Herman, 2000; Yura et al., 2000). Thus, the enhanced synthesis and marked stabilization of σ^{32} that are observed upon a sudden temperature upshift represent two distinct regulatory events that involve different signaling pathways: one that is directly mediated by temperature and one that is mediated by chaperones and proteases. These distinct σ^{32} regulatory mechanisms are discussed below.

The intracellular levels of σ^{32} and its activity are regulated by temperature at several levels. While the majority of this regulation is posttranscriptional, low levels of σ^{32} mRNA are maintained in the cell under normal physiological conditions (i.e., in the absence of thermal stress) (Craig and Gross, 1991; Straus et al., 1987). This ensures that basal levels of σ^{32} are present to initiate the heat shock response when it is needed. At least four promoters regulate

the transcription of the *rpoH* gene, three of them are regulated by σ^{70} and one is regulated by σ^{E} (Erickson et al., 1987; Nagai et al., 1990), the latter of which regulates the expression of σ^{32} in response to very high temperatures (i.e., exceeding 42°C) (discussed below) (Erickson et al., 1987; Wang and Kaguni, 1989). However, the potential regulatory roles of these various promoters in σ^{32} transcriptional regulation are not fully understood, and will not be discussed further. It is important to mention that the *rpoH* mRNA serves not only as a messenger but also as a thermal sensor and regulator of translation (Arsene et al., 2000; Guisbert et al., 2004; Herman, 2000; Yura et al., 2000). At low temperatures, the secondary structure of *rpoH* mRNA is folded into a structure that inhibits translational initiation by blocking ribosome access, thus limiting expression of the heat shock genes when they are not needed (Morita et al., 1999a, b). At higher temperatures, however, this inhibitory mRNA structure is disrupted, which permits ribosome access to the translational start site, efficient production of σ^{32} protein, and subsequent induction of the heat shock regulon (Figure 10) (Morita et al., 1999a, b).

In addition to translational induction during temperature upshift mediated by changes in *rpoH* mRNA secondary structure, significant stabilization of σ^{32} protein occurs. This stabilization of σ^{32} protein is transient and occurs only during the initial phase (first 4–5 min) of heat shock, which is followed by rapid destabilization (Straus et al., 1987). Sigma 32 stability (i.e., turnover) and activity are regulated by its interaction with other proteins. Specifically, *E. coli* uses a network of chaperones and proteases to control both the stability and activity of σ^{32} (Arsene et al., 2000; Guisbert et al., 2004; Herman, 2000; Yura et al., 2000). In this regard, core RNA polymerase and chaperone proteins compete with each other for binding to σ^{32}. Whether σ^{32} interacts with RNA polymerase or chaperone proteins depends upon the thermal stress status (i.e., protein folding state) of the cell. Binding of σ^{32} to RNA polymerase or chaperones has opposing effects on RpoH stability. Specifically, chaperone binding results in σ^{32} degradation, whereas RNA polymerase binding stabilizes and activates σ^{32} (Arsene et al., 2000; Guisbert et al., 2004; Herman, 2000; Yura et al., 2000).

At low temperatures (when unfolded proteins are low and there is a decreased need for protein-folding agents), σ^{32} is inactive and unstable because of its association with the chaperone proteins DnaK, DnaJ, and others, like GroEL/S (Arsene et al., 2000; Guisbert et al., 2004; Herman, 2000; Yura et al., 2000). Binding of σ^{32} by these chaperones inactivates σ^{32} by preventing it from interacting with RNA polymerase, and it renders σ^{32} unstable by promoting its degradation by a set of proteases, including FtsH, HslVU, ClpP, and Lon (Arsene et al., 2000; Guisbert et al., 2004; Herman, 2000; Yura et al., 2000). This negative regulation (i.e., destabilization) of σ^{32} by chaperone binding prevents induction of heat shock genes in the absence of thermal stress. However, during periods of thermal stress (when there is an increased need for protein-folding agents) high levels of misfolded and unfolded proteins accumulate in the cytoplasm and are able to titrate chaperones away

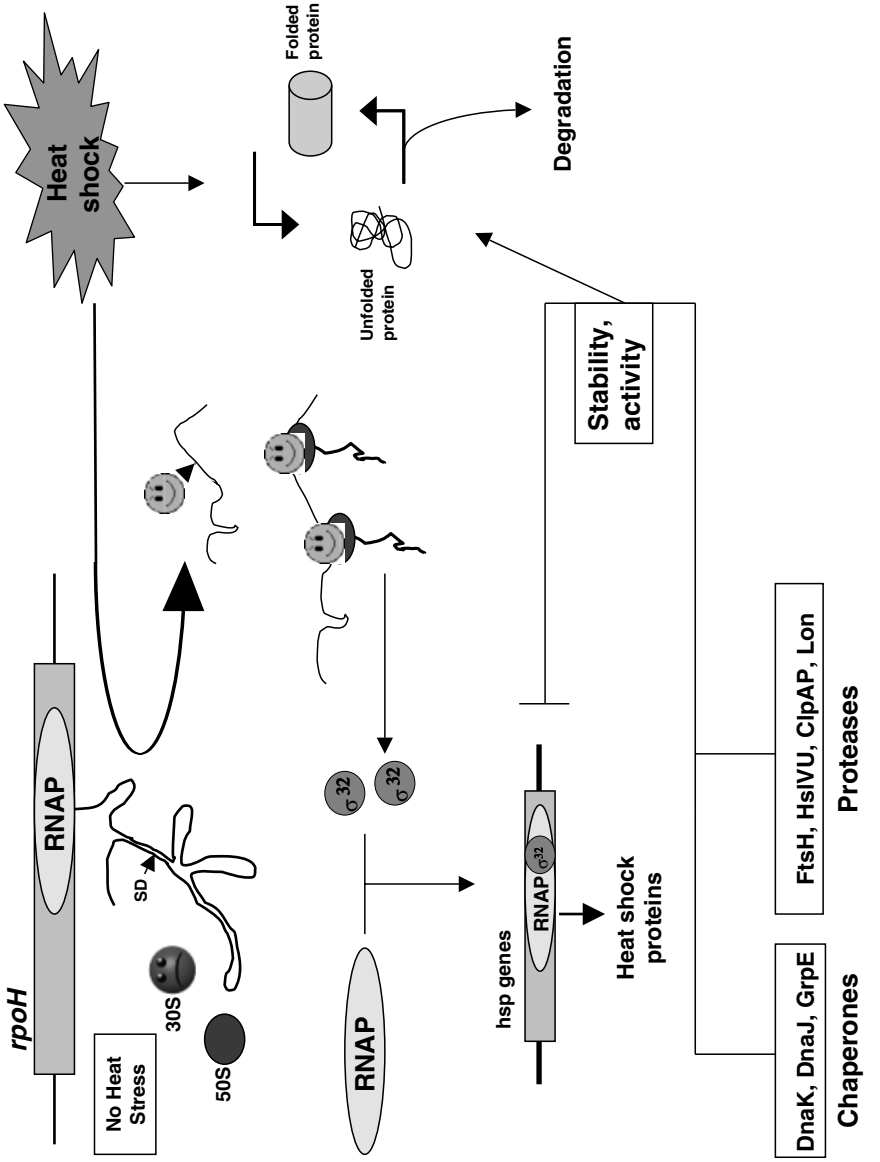

FIGURE 10. Hypothetical regulatory circuits of the σ^{32} regulon in *Escherichia coli*. *Translation*: Under conditions of normal physiological growth (no thermal stress) the *rpoH* mRNA secondary structure is in a conformation that is inaccessible to ribosomes. However, during heat stress the inhibitory mRNA secondary structure melts and the ribosome-binding site is accessible for translation. *Protein stability*: Sigma 32 is normally held in an inactive state in complex with the chaperone DnaK. Upon heat stress, unfolded proteins compete for binding to DnaK, and σ^{32} is released to activate RNA polymerase transcription of the cytoplasmic stress response genes. RNAP, RNA polymerase core; SD, Shine–Dalgarno sequence (i.e., ribosomal binding site). (Adapted from Yura et al., 2000 and reprinted with permission from the American Society for Microbiology, Bacterial Stress Responses, Storz and Hengge-Aronis, Editors, 2000, ASM Press, Washington, D.C.).

from σ^{32} so that they can instead bind and help repair misfolded proteins. Consequently, there are insufficient levels of the proteolytic machinery to degrade σ^{32}. The now active, stable, chaperone-free form of σ^{32} can bind to core RNA polymerase and initiate transcription of the heat shock genes (initiation phase). Thus, in response to a rapid upshift in temperature, σ^{32} stability and activity are increased, which accounts for the immediate induction of HSP synthesis. However, σ^{32} stabilization is transient and is limited only to the initial phase of the heat shock response, when there is an urgent need for protein-folding agents (Fig. 10).

During the adaptation or recovery phase following temperature upshift (or immediately after temperature downshift), there is decreased need for protein-folding agents; thus the stability and activity of σ^{32} is decreased. What is responsible for the decrease in σ^{32} stability and activity? It is believed to be due to the increased accumulation of chaperones and proteases that occurs shortly after temperature upshift, which causes these proteins to reach high enough levels to bind to, and destabilize, σ32 (Arsene et al., 2000; Guisbert et al., 2004; Herman, 2000; Yura et al., 2000). This results in a decrease in σ^{32} levels and activity and the heat shock response is turned off. Thus, initial stabilization of σ^{32} results from titrating this sigma factor away from the chaperones because of heat-induced accumulation of unfolded or misfolded proteins in the cytoplasm, thus allowing σ^{32} to bind core RNA polymerase and induce the heat shock regulon. Alternatively, subsequent destabilization of σ^{32} results from accumulation of chaperones and proteases caused by their increased expression from σ^{32} promoters (Figure 10).

Gram-positive bacteria use a variety of different and diverse regulatory strategies for heat shock regulation as compared to most Gram-negative bacteria. The contribution of these regulatory strategies to the heat shock response varies in different Gram-positive bacteria. The organism that has been used most extensively for studying the heat shock response in Gram-positive bacteria is the model microbe *B. subtilis* (Hecker and Engelmann, 2000; Helmann et al., 2001; Price, 2000; Price et al., 2001; Rosen and Ron, 2002; Yura et al., 2000). This organism uses a variety of proteins to regulate the expression of HSPs, including:

(1) The general stress response regulator, σ^{B} (discussed above) (Price, 2000; Price et al., 2001; Rosen and Ron, 2002; Yura et al., 2000).
(2) The chaperones DnaK-J-Grp-E and GroEL-GroES, whose transcription is regulated by the major vegetative sigma factor, σ^{A}, and is negatively controlled by a protein repressor encoded by the *hrcA* gene (Price, 2000; Price et al., 2001; Rosen and Ron, 2002; Yura et al., 2000). The HrcA protein is a sequence-specific repressor protein that exerts its effect by binding to a *cis* element in the operator region of heat shock genes called controlling inverted repeat of chaperone expression (CIRCE). The CIRCE element is highly conserved and consists of a well-conserved 9 bp inverted repeat with a 9 bp sequence. The CIRCE/HrcA regulon in *B. subtilis* is normally repressed under normal physiological growth

conditions (i.e., no thermal stress or other stresses that can cause formation of non-native proteins) by the HrcA repressor. However, this regulon can be heat-induced by inactivating the repressor, which is mediated by the activity of chaperones like GroEL-GroES and DnaK-DnaJ (Price, 2000).

(3) Proteins belonging to the CtsR regulon encode the highly conserved Clp proteases that are negatively regulated by the CtsR repressor (Price, 2000; Price et al., 2001; Rosen and Ron, 2002; Yura et al., 2000). The CtsR repressor exerts its effect by binding to a conserved heptameric *cis* element in the promoter region. The transcription of these genes is complex and is regulated by both the vegetative sigma factor σ^A and the stress sigma factor σ^B. The induction pattern of the genes in this group is different for different genes and also depends on the specific stress condition.

(4) Several groups of heat-inducible genes (including *lon*, *ftsH,* and *htpG*) that are controlled by undefined mechanisms (Price, 2000; Price et al., 2001; Rosen and Ron, 2002; Yura et al., 2000).

2.3.1. Role of Heat Shock Proteins in Bacterial Pathogenesis

Bacteria encounter heat stress, and other stresses that regulate HSPs, during their pathogenic life cycles within the infected host. These stresses serve as key signals to increase the synthesis of HSPs (both in the cytoplasm and periplasm), thus allowing the pathogen to survive the hostile environment encountered during infection. Indeed, it is well known that bacterial HSPs are induced during infection and not only play a role in the proper folding of intracellular proteins but also mediate bacterial adherence and invasion, regulate the expression of virulence genes, and even serve as antigens that can confer protective immunity against infection by certain pathogens (Buchmeier and Heffron, 1990; Charpentier et al., 2000; Fernandez et al., 1996; Gaillot et al., 2000; Kwon et al., 2003; Nair et al., 2000; Pederson et al., 1997; Schoel, 1994; VanBogelen, 1987; Webb et al., 1999). Some bacterial HSPs are themselves virulence factors, while others affect pathogenesis indirectly, by increasing bacterial resistance to host defenses or regulating virulence genes. For example, depletion of HSPs, like the Clp cytoplasmic proteases and the DegP periplasmic proteases, has been shown to affect the sensitivity of bacteria to thermal and environmental stresses, and is essential for the virulence of many bacterial pathogens, including *Klebsiella pneumoniae* (Cortes et al., 2002), *Brucella abortus* (Elzer et al., 1996), *S. typhimurium* (Johnson et al., 1991), *Y. enterocolitica* (Li et al., 1996), *L. monocytogenes* (Gaillot et al., 2000), *S. flexneri* (Purdy et al., 2002); *S. pyogenes* (Jones et al., 2001), and *S. pneumoniae* (Kwon et al., 2003; Sebert et al., 2002). In recent years, several studies have reported the characterization of HSPs produced by oral microorganisms, like *Actinobacillus* spp., *Bacteroides* spp., *Prevotella* spp., *Porphyromonas gingivalis*, *S. mutans*, *Candida albicans*, and *Treponema denticola* (Goulhen et al., 2003). Like the HSPs involved in other diseases, these stress-induced proteins may contribute to the pathogenic process of oral infections, such as dental caries, periodontal disease, and stomatitis.

2.4. Sigma 24—Periplasmic Stress

Gram-negative bacteria have two compartments: the cytoplasm and the cell membrane, which is composed of the inner membrane, periplasm, and outer membrane; and they use separate cellular stress response systems to serve each compartment (Alba and Gross, 2004). In Section 2.3, we discussed how cytoplasmic stress results in the activation of the heat shock sigma factor, σ^{32}. In this section, we will discuss the use of the extracytoplasmic sigma factor, σ^{24} (σ^{E}), to respond to thermal stress in the cell envelope, and its role in bacterial pathogenesis.

The bacterial cell envelope is directly exposed to the external milieu and serves as a vital shield to protect the organism against environmental stresses, as well as determining cell morphology and structural integrity. The envelope contains a wide variety and large number of bacterial proteins that play important roles in maintaining cell viability, including solute transport, protein translocation, lipid biosynthesis, and oxidative phosphorylation (Kadner, 1996; Nikaido, 1996; Oliver, 1996; Park, 1996). Thus, the cell must have repair mechanisms for these envelope proteins to ensure their function and sustain cell viability. This is especially important for bacterial pathogens, where the harsh environment encountered in the infected host is likely to affect the membrane structure of the cell wall of the organism, which is often the first structure that comes in contact with the host during the infection process. Extracytoplasmic stresses often result in the generation of unfolded or misfolded proteins in the membrane and/or periplasm, which must be repaired to ensure cell viability. A major mechanism used by bacteria to repair damage to bacterial envelope proteins and restore envelope integrity is the use of extracytoplasmic sigma factors. Many bacteria have multiple ECFs (e.g., *S. coelicolor* has at least 60 ECF sigma factors) (Bentley et al., 2002). Despite the impressive number of bacterial ECF members, they all share three common themes: (1) they regulate the response to extracytoplasmic stresses; (2) they are regulated by antisigma and/or anti-antisigma factors; and (3) they typically control a relatively small set of genes (Bashyam and Hasnain, 2004; Missiakas and Raina, 1998; Silhavy, 2000).

Sigma 24 (σ^{E}) is the best-characterized ECF and will be used as the model for discussion in this section. Sigma 24 was originally discovered in *E. coli* as a sigma factor important for the regulation of σ^{32} activity in response to extreme heat shock conditions (temperatures exceeding 42˚C). As previously mentioned, one of the four *E. coli rpoH* promoters that controls σ^{32} mRNA expression is regulated by σ^{24} under conditions of extremely high temperature. Like the cytoplasmic heat shock response, σ^{24} is activated by excessive levels of misfolded and/or unfolded proteins that are a result of exposure of the bacterial cell to stresses like extreme heat and ethanol (Erickson et al., 1987; Rouviere et al., 1995). However, unlike the cytoplasmic heat shock response, σ^{24} responds to aberrantly folded proteins in the bacterial cell envelope, rather than the cytoplasm (Mecsas et al., 1993). In particular, misfolded

outer-membrane proteins (OMPs) are believed to be the specific envelope stress signal that activates σ^E (Mecsas et al., 1993). During normal physiological growth conditions or moderate increases in temperature, (when unfolded envelope proteins are low), basal levels of σ^{24} are produced by the bacterial cell. These low levels of σ^{24} are necessary for maintenance of the cell envelope homeostasis and to ensure that basal levels of σ^{24} are present to initiate the heat shock response when it is needed. Alternatively, during times of extreme thermal stress, when high levels of misfolded and unfolded proteins accumulate in the cell envelope, σ^{24} levels are elevated to repair these proteins. The repair functions of σ^{24} result from its ability to bind core RNA polymerase and direct the transcription of genes that encode products that are mainly targeted to the cell envelope, including periplasmic proteases, folding catalysts, key enzymes in the biosynthesis of cell envelope constituents, and a number of lipoproteins (Alba and Gross, 2004; Raivio and Silhavy, 2001).

Although σ^{24} (σ^E) is of central importance for maintaining the integrity of the bacterial cell envelope during the stress response, our knowledge is incomplete regarding its regulation. Because σ^E is a cytoplasmic protein, it is unable to directly sense the levels of damaged proteins in the cell envelope. How then does the bacterial cell communicate the presence of aberrantly folded OMPs in the cell envelope to σ^E in the cytoplasm? As shown in Figure 11 for *E. coli*, membrane perturbations caused by misfolded or unfolded OMPs are communicated to σ^E through an inner membrane–bound protein called RseA. RseA is an antisigma factor that contains a single transmembrane-spanning segment, which has an N-terminal cytoplasmic domain and a C-terminal periplasmic domain. The N-terminal domain of RseA binds to σ^E and inhibits its activity under nonstress conditions, while its C-terminal domain binds RseB, a protein that enhances the affinity of RseA for σ^E, and thereby contributes to the inactivation of σE (Ades et al., 1999; Alba and Gross, 2004; Collinet et al., 2000; De Las Penas et al., 1997; Missiakas and Raina, 1998). Thus, RseA acts as a negative regulator of σ^E function by preventing its interaction with core RNA polymerase. By binding to RseA, RseB acts to further reinforce this negative interaction (Campbell et al., 2003). Clearly, the interaction between RseA and σ^E must be disrupted for σ^E to bind to core RNA polymerase and effectively initiate transcription of the periplasmic heat shock genes. The cell accomplishes this task through the action of two inner-membrane proteases, DegS and YaeL, which are responsible for cleaving σ^E from RseA (Ades et al., 1999; Alba et al., 2002; Kanehara et al., 2002; Rosen and Ron, 2002; Walsh et al., 2003).

The cell regulates the activity of these proteases by monitoring the level of misfolded and unfolded OMPS in the cell envelope. In nonstressed cells, DegS is normally in an inactive conformation because its PDZ domain interacts with its protease domain and prevents it from binding RseA. In stressed cells, however, membrane perturbations caused by aberrantly folded OMPs stimulate DegS-mediated proteolytic degradation of RseA, thus alleviating the inhibitory interaction between σE and RseA. How do unfolded OMPs

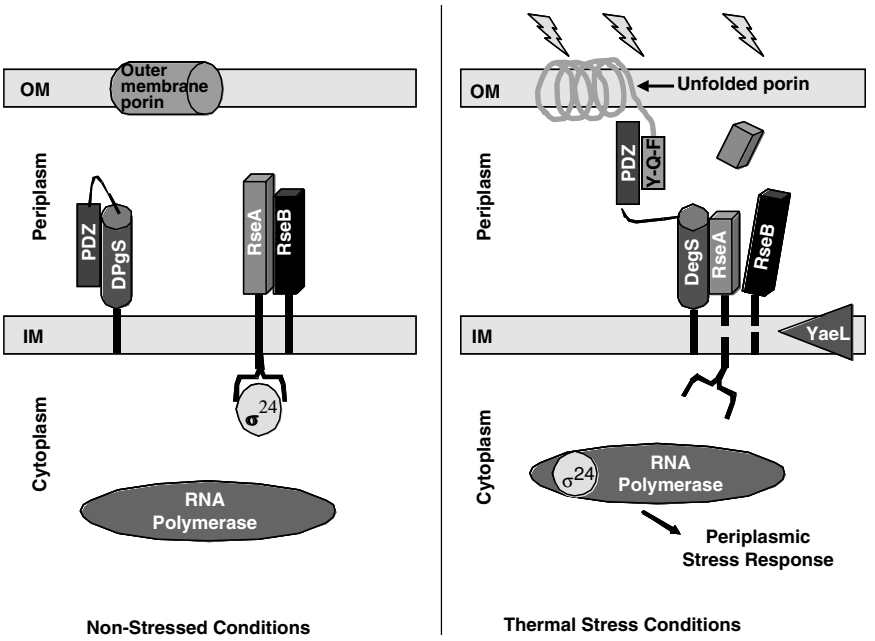

FIGURE 11. The bacterial heat stress signaling pathways. *Periplasm*: Under nonstress conditions, σ^{24} is inactive and sequestered by RseA and RseB. DegS, the periplasmic stress sensor, is normally inactive, with its PDZ domain inhibiting its protease domain. Recognition by the PDZ domain of the unfolded C-terminal YQF motifs of outer membrane porins relieves inhibition of the DegS protease. Activated DegS cleaves RseA, which is further cleaved by YaeL. The cytoplasmic domain of RseA is then destabilized, releasing σ^{24} to induce expression of the periplasmic stress response genes. Under conditions of extreme heat (temperatures above 42°C) σ^{24} also regulates the expression of σ^{32}. (Adapted from Young and Hartl, 2003.)

trigger the activiation of DegS proteolytic activity? This process occurs through the use of the PDZ domain of DegS to recognize and bind YQF motifs of unfolded OMPs (Walsh et al., 2003). The YQF peptide sequences of OMPs are found in their C-terminal region and are normally concealed within a properly folded protein (i.e., under nonstress conditions). However, this peptide sequence becomes accessible when OMPs unfold due to thermal stress and can then be recognized by the PDZ domain of the DegS protease (Walsh et al., 2003). Binding of DegS to the YQF motif of unfolded OMPs results in a conformational change in the protein that activates the protease function of DegS. Activated DegS then cleaves RseA, which is further cleaved by YaeL. The degradation of RseA results in the release of σ^{E} from its inhibitory complex with the antisigma factor. Once released, the active σ^{E} can then bind to core RNA polymerase and initiate transcription of specific

heat shock genes, including OMP folding and degrading factors FkpA and DegP, *rpoH* and *rpoE* (encoding σ^{32} and σ^{E}, respectively), the *rseA* and *rseB* genes (encoding an inner membrane and periplasmic protein, respectively), and *rseC* (a gene of unknown function) (Alba and Gross, 2004; Silhavy, 2000). It is important to note that σ^{E}, RseA, and RseB are all members of an operon that is transcribed by σ^{E}-containing RNA polymerase holoenzyme. This autoregulatory loop provides the bacterial cell with the ability to tightly control σ^{E} activity, since upon activation both σ^{E} and its inhibitors are transcribed together (Alba and Gross, 2004; Silhavy, 2000).

2.4.1. Role of σ^{24} in bacterial pathogenesis

Sigma 24 (σ^{E}) plays a role in the virulence of a variety of different bacteria, including *S. typhimurium*, *Haemophilus influenzae*, *V. cholerae*, *P. aeruginosa*, *M. tuberculosis*, *and E. coli* (Ando et al., 2003; Craig et al., 2002; Deretic et al., 1994; Humphreys et al., 1999; Kovacikova and Skorupski, 2002; Manganelli et al., 2004a; Testerman et al., 2002; Yu et al., 1996).

In *S. typhimurium* and *H. influenzae*, σ^{E} is necessary for intracellular survival within macrophages (Craig et al., 2002; Humphreys et al., 1999; Testerman et al., 2002). In *V. cholerae* σ^{E} is required for virulence and intestinal colonization in the mouse model of infection (Kovacikova and Skorupski, 2002). In *P. aeruginosa*, the σ^{E} homolog, AlgU, confers resistance to osmotic, oxidative, and heat stresses and phagocytic killing, and also plays a role in the development of cystic fibrosis as it regulates the production of alginate, a secreted polysaccharide required for bacterial persistence in human lungs (Deretic et al., 1994; Martin et al., 1994; Yu et al., 1996; Yura et al., 1993). Interestingly, two sigma factors, σ^{E} (AlgU) and σ^{54}, control the mucoid phenotype in *P. aeruginosa* that is observed during chronic respiratory infections in cystic fibrosis patients (Boucher et al., 2000; Deretic et al., 1994; Yu et al., 1996).

Loss-of-function mutations in σ^{E} result in an increased susceptibility of a variety of bacteria to oxidative stress, including the pathogens *Salmonella*, *Pseudomonas*, and *Mycobacterium* spp. (Martin et al., 1994; Testerman et al., 2002; Wu et al., 1997). For example, an *rpoE* mutant in *S. typhimurium* exhibits reduced viability during oxidative stress, reduced ability to survive stationary phase (starvation), and is attenuated for virulence in a mouse model of infection (Testerman et al., 2002). In this regard, the σ^{E} regulon has been shown to compliment the RpoS (σ^{S}) regulon in allowing *S. typhimurium* to withstand oxidative stress during stationary phase in vitro and to resist the generation of reactive oxidative intermediates by innate host defenses during the infection process (Testerman et al., 2002). Indeed, the σ^{E} regulon complements the RpoS regulon in *Salmonella* and allows the organism to better survive the oxidative stress associated with stationary phase, since mutants lacking both σ^{E} and σ^{S} are more sensitive to stationary phase oxidative stress conditions than either mutant alone (Testerman et al., 2002).

Sigma E also plays an important role in *M. tuberculosis* pathogenesis, as σ^E mutants in this pathogen (1) are attenuated for virulence (Ando et al., 2003; Manganelli et al., 2004a); (2) show decreased numbers in the lungs of infected mice as compared to the wild-type strain (Manganelli et al., 2004a); (3) induce formation of lung granulomas in infected mice with different structural characteristics than those caused by the wild-type strain (Manganelli et al., 2004a); and (4) exhibit decreased viability in both activated and unactivated macrophages (Manganelli et al., 2001). Not surprisingly, σ^E expression increases following phagocytosis by human and mouse macrophages (Graham and Clark-Curtiss, 1999; Jensen-Cain and Quinn, 2001).

Recently, σ^E has also been implicated in the virulence of extraintestinal *E. coli* strains that cause meningitis, sepsis, urinary tract infection, and other infections outside the bowel (Redford et al., 2003). In this study, an *E. coli* strain containing a loss-of-function mutation in DegS was attenuated for virulence in peritoneal and urinary tract infections in mice. Virulence was restored upon plasmid complementation of *degS* into the mutant strain. While the exact nature of the virulence attenuation is not known, DegS does plays a major role in regulating the activity of σ^E; thus, these findings suggest that σ^E may be required for virulence in *E. coli*.

2.5. Sigma 28—Motility and Chemotaxis Genes

Originally discovered in *B. subtilis*, σ^{28} (FliA, σ^F, σ^D) regulates the expression of genes required for flagellar assembly and chemotaxis in a variety of bacteria, including the pathogens *L. pneumophila, S. typhimurium, E. coli, Y. enterocolitica, Helicobacter pylori, T. maltophilum, P. aeruginosa, V. cholerae, Chlamydia trachomatis, Proteus mirabilis* and *Campylobacter jejuni* (Belas and Flaherty, 1994; Carrillo et al., 2004; Helmann and Chamberlin, 1987; Heuner et al., 1995, 2002; Ionescu et al., 1989; Iriarte et al., 1995; Leying et al., 1992; Starnbach and Lory, 1992). (As discussed later in this chapter, another sigma factor also plays a role in the flagellar synthesis of some bacteria.) Bacterial flagella are remarkably complex structures and their production requires a significant energy expenditure; thus, intricate regulatory mechanisms are used to control flagellar structural gene expression. In Gram-negative motile bacteria, flagellar biosynthesis is transcriptionally regulated in a hierarchical cascade, with genes expressed in the order in which they are required for the assembly of the flagellum. Expression of each class is a prerequisite for expression of the following class in the hierarchical cascade, thereby allowing coordinated expression starting from the cell envelope and extending extracellularly to the tip of the flagellum (Aldridge and Hughes, 2002; Chilcott and Hughes, 2000).

In *E. coli* and *S. typhimurium*, for example, the flagellar regulon comprises more than 50 genes that are divided among 17 operons (Macnab, 1996). These operons are transcribed in three temporal phases of gene expression—early, middle, and late (corresponding to class 1, 2, and 3 genes, respectively)—

that control the morphological development of the flagellar structure (Aldridge and Hughes, 2002; Chilcott and Hughes, 2000; Komeda, 1986; Kutsukake et al., 1990). The early (class 1) genes are transcribed from σ^{70}-dependent promoters and consist of *flhC* and *flhD*, which encode the master regulators of the flagellar cascade FlhC and FlhD, respectively, and are required for expression of the remaining flagellar genes. The bacterial cell senses specific environmental signals to commence expression of the *flhC* and *flhD* genes, and this is a key regulatory point where the decision is made whether to initiate or prevent flagellar biosynthesis. If the cell receives the stimulatory signals needed to synthesize flagella, the FlhC and FlhD proteins are produced and subsequently form a multimeric complex that promotes the σ^{70}-dependent transcription of the middle (class 2) genes (Aldridge and Hughes, 2002; Chilcott and Hughes, 2000; Liu and Matsumura, 1994). The middle genes encode proteins forming the flagellar hook/basal body complex, the flagellar secretory export complex (which is a type III secretion system or T3SS), and two competing regulatory proteins, FliA (σ^{28}) and FlgM (an anti-sigma factor that inhibits σ^{28} activity) (Aldridge and Hughes, 2002; Chadsey et al., 1998; Chilcott and Hughes, 2000; Macnab, 1996; Ohnishi et al., 1990, 1992). Sigma 28 is required for transcription of the late (class 3) genes that are incorporated last into the growing flagellum, including the flagellin subunits that form the external flagellar filament, motor torque–generating subunits, and chemotaxis proteins (Aldridge and Hughes, 2002; Chilcott and Hughes, 2000). The role of FlgM is to ensure that the class 3 genes are not transcribed until the complete flagellar structure is formed (i.e., after the hook/basal body completion), thus providing the bacterial cell with a mechanism to sense the state of flagellar synthesis and adjust its transcription accordingly (Aldridge and Hughes, 2002; Chilcott and Hughes, 2000). After formation of a complete basal body and hook structure, the FlgM antisigma factor is secreted through this nascent structure to the external (extracellular) environment, thus decreasing intracellular FlgM levels and relieving repression of σ^{28}, which allows transcription of the class 3 genes (Figure 12) (Aldridge and Hughes, 2002; Chilcott and Hughes, 1998, 2000; Karlinsey et al., 2000; Saito et al., 1998).

While the flagellar transcriptional cascade described above is widely conserved in a variety of bacteria, some bacteria use both σ^{54} and σ^{28} to control transcription of flagellar genes, including *H. pylori*, *V. cholerae*, *C. jejuni*, and *P. aeruginosa* (Colland et al., 2001; Josenhans et al., 2002; Jyot et al., 2002; Klose and Mekalanos, 1998; Klose et al., 1998; Prouty et al., 2001; Spohn and Scarlato, 1999; Starnbach and Lory, 1992; Totten et al., 1990). It is clear from such a complex transcriptional regulation of flagellar genes that motility plays an important role in the bacterial cell cycle. While an extremely energy-draining expenditure for the bacterial cell, the production of flagella and the resulting motility could confer an important survival advantage for a cell to reach a food source ahead of competitors and/or move away from agents that could damage the cell. Regulation of the entire flagellar regulon

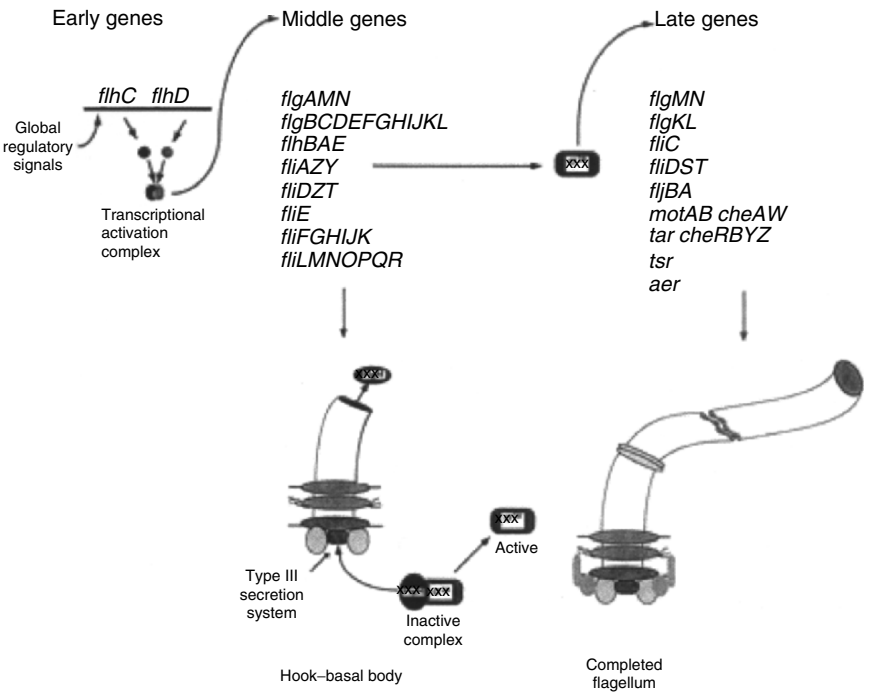

FIGURE 12. Flagellar transcriptional hierarchy coupled to flagellar assembly. There are more than 50 genes in the flagellar and chemotaxis regulon. These genes are transcribed in operons of three temporal classes: early, middle, and late. The early genes are included in the master flagellar operon, *flhDC*. The FlhC and FlhD proteins form a heteromultimeric complex that directs σ^{70}-dependent transcription of class 2 promoters of the middle and some late genes. The middle operons encode structural and assembly proteins required for the biosynthesis of the flagellar motor intermediate structure, also known as the hook/basal body proteins. In addition to hook/basal body proteins, two competing regulatory proteins, FlgM and FliA, are also transcribed from class 2 promoters. The *fliA* gene encodes an alternative σ factor, σ^{28}, which is specifically required for class 3 promoter transcription. Genes whose products are required late in flagellar assembly, including the external filament (flagellar propeller), are primarily transcribed from class 3 promoters. The FlgM protein binds to σ^{28} directly to prevent class 3 promoter transcription until after hook/basal body completion. Once the hook/basal body is complete, FlgM is secreted from the cell to free σ^{28}, and class 3 transcription occurs. In this way, the external filament (propeller) is not made until there is a motor (hook/basal body) for it to polymerize onto. A number of late flagellar genes, including *flgK*, *flgL*, *flgM*, *flgN*, *fliD*, *fliS*, and *fliT*, are expressed in both middle and late operons. (Reprinted with permission from the American Society for Microbiology, Chilcott and Hughes, 2000, Microbiol Mol Biol Rev, 64:694–708).

is quite complex and in addition to the described transcriptional regulation, it includes translational and posttranslational mechanisms. However, a comprehensive overview of the entire flagellar regulatory network is not within the scope of this chapter. There are several excellent recent reviews in this area to which the reader is referred (Aldridge and Hughes, 2002; Chilcott and Hughes, 2000).

On an interesting note, sequencing of the *fliA* genes from *E. coli* and *S. typhimurium* revealed that the σ^{28} protein is considerably smaller than the primary σ^{70} because it lacks region 1, whereas regions 2, 3, and 4 are conserved between the two sigmas (Arnosti and Chamberlin, 1989; Dombroski et al., 1992, 1993; Liu and Matsumura, 1995; Ohnishi et al., 1992). As described earlier in this chapter, the σ^{70} family contains four conserved regions of homology that can be further subdivided within a given region (Paget and Helmann, 2003). Region 1 (which is absent in σ^{28}) prevents σ^{70} from binding to promoter sequences when it is not bound to RNA polymerase. In free σ^{70} (i.e., not bound to RNA polymerase) region 1 interacts with region 4 (which contains the 35 promoter-binding domain). Since region 1 is not present in σ^{28}, how is free σ^{28} kept from binding to promoter DNA? The answer lies in region 4 of σ^{28}, which is prevented from binding DNA when free from RNA polymerase by interaction with the antisigma factor FlgM (Chadsey et al., 1998; Iyoda and Kutsukake, 1995).

2.5.1. Role of σ^{28} in Bacterial Pathogenesis

Sigma 28 has been implicated in the virulence of several bacterial pathogens. For example, *L. pneumophila*, the causative agent of a severe form of pneumonia called Legionnaire's disease, is a pathogen that cycles between an intracellular lifestyle in amoeba (and other protozoa) in the environment and in alveolar macrophages in the infected human lung. A *fliA* mutant in *L. pneumophila* was shown to be defective in its ability to multiply intracellularly within host amoeba cells. This intracellular replication defect was fully complemented by introduction of the wild-type *fliA* gene back into the mutant strain (Heuner et al., 2002). Moreover, using global transcriptional and proteomic analysis in combination with animal models of virulence, it was determined that the disease-causing potential of the gastrointestinal pathogen *C. jejuni* is regulated by changes in expression of genes that are controlled by σ^{28} (Carrillo et al., 2004). Specifically, a *fliA* mutant of *C. jejuni* was shown to regulate the expression of specific flagellar transcripts and virulence factors, and exhibited significantly reduced ability to colonize young chicks and invade into tissue culture cells. In this regard, it is interesting to note that an earlier study reported that σ^{28} regulates the transcriptional expression of genes associated with the *Salmonella* pathogenicity island I (SPI-I)-encoded type III secretion system (T3SS), as well as phenotypes associated with these genes (Eichelberg and Galan, 2000). The SPI-I T3SS encodes genes that are required for invasion into intestinal epithelium, cytotoxicity of macrophages,

and bacterial virulence (see Chapter 9). Serovar Typhi and Typhimurium strains carrying loss-of-function mutations in σ^{28} exhibited reduced expression of SPI-I invasion genes and diminished ability to invade cultured epithelial cells and induce macrophage cytotoxicity (Eichelberg and Galan, 2000). However, the authors of this study caution that dissecting the role of flagella in *Salmonella* virulence should be interpreted with care, considering the connection between the regulation of flagellar genes and genes associated with T3SS. The regulatory connection between these systems seems logical, since the flagellar export and T3SSs are evolutionarily and functionally related (Galan and Collmer, 1999). Thus, the effect of σ^{28} on invasion gene expression and associated phenotypes in *Salmonella* may be not direct (i.e., not due to motility per se) but rather a consequence of the influence of this system on SPI-I gene expression (Eichelberg and Galan, 2000). While the coordinate expression of virulence and flagellar genes has also been documented for other bacteria (Allison et al., 1992; Gygi et al., 1995), it is not currently known if there is a connection between the regulation of flagellar and T3SS genes in bacteria other than *Salmonella*.

2.6. *In a Class by Itself: σ^{54}—Nitrogen Metabolism and So Much More!*

Sigma factor 54, RpoN (encoded by the *rpoN* gene), does not share obvious homologies with other known sigma factors and works in concert with a special class of transcriptional activators to control the expression of many genes in response to nutritional and environmental conditions (Buck et al., 2000; Studholme and Buck, 2000; Studholme and Dixon, 2003). First identified in *E. coli*, σ^{54} is now known to be widely distributed throughout phylogenetically diverse bacteria (both Gram-positive and Gram-negative), which underscores its biological importance (Studholme and Buck, 2000). Indeed, σ^{54} has been found in a variety of bacterial pathogens, including *H. pylori*, *Bordetella pertussis*, *Chlamydia* spp., *Vibrio* spp., *Salmonella* spp., *Pseudomonas* spp., *Y. pestis*, *P. mirabilis*, *Neisseria gonorrhoeae*, and the spirochetes *B. burgdorferi* and *T. pallidium* (Studholme and Buck, 2000). While best known for its regulation of nitrogen metabolism, σ^{54} is also required for a wide variety of other functions in bacteria including carbon source utilization, RNA modification, chemotaxis, flagellar synthesis, pilin, electron transport, response to heat and phage shock, expression of alternative sigma factors, and bacterial virulence (Studholme and Buck, 2000). In this respect, σ^{54} (RpoN) differs from a lot of the other alternative sigma factors (excluding RpoS and σ^{B}) in that it regulates the expression of genes that encode a large repertoire of diverse functions. However, the diversity of functions regulated by σ^{54} varies dramatically between different bacteria. Many bacteria possess a single copy of *rpoN*, which is constitutively expressed and is not required for survival and growth under normal physiological conditions (Buck et al., 2000).

As described above, σ^{54} (i.e., σ^{N}, RpoN, NtrA), is quite distinct from all other known bacterial sigma factors, which belong to the σ^{70} family. Specifically, σ^{54} differs both in its amino acid sequence and in transcription mechanism from the σ^{70} class (Buck et al., 2000; Studholme and Buck, 2000; Studholme and Dixon, 2003). Promoters recognized by σ^{54}-containing RNA polymerase are unique in having conserved promoter elements centered at –24 and –12 with respect to the transcriptional start site, instead of the typical –35 and –10 locations recognized by σ^{70} promoters (Buck et al., 2000; Studholme and Buck, 2000; Studholme and Dixon, 2003). Moreover, unlike σ^{70} promoters, expression of genes from σ^{54}-dependent promoters is absolutely dependent upon a special type of activator protein. These activator proteins belong to the large AAA+ (ATPase associated with a variety of cellular activities) protein family that use nucleotide binding and hydrolysis to remodel their substrates (Neuwald et al., 1999; Popham et al., 1989; Rombel et al., 1998; Wedel and Kustu, 1995). The binding sites for these activators (called enhancers) are usually located far from the transcription start site (either upstream or downstream) and involve DNA looping. For some σ^{54} promoters, the looping out of the intervening DNA (which facilitates interaction between the activator and RNA polymerase) is facilitated by a DNA-bending protein like integration host factor (IHF) or arginine repressor (ArgR) (Bertoni et al., 1998; Lu and Abdelal, 1999) (Fig. 13). While transcriptional activators can also regulate the activity of σ^{70}-containing holoenzyme, in this case, the activators bind adjacent to the polymerase site and touch the enzyme without looping (Gralla, 1991). A distinctive feature of the activator proteins important for controlling expression from σ^{54} promoters is that they possess an ATPase activity. This ATPase activity is used to overcome the block to DNA melting within the closed promoter complex and allows transcription to initiate (Buck et al., 2000; Studholme and Buck, 2000; Studholme and Dixon, 2003). While core RNA polymerase containing σ^{70} is sufficient for open complex formation, σ^{54}-containing holoenzyme can only drive open complex formation with the help of these special activator proteins that use ATP hydrolysis to catalyze open complex formation (Figure 13). Indeed, the main factor that controls the expression of σ^{54}-dependent genes is the modulation of the expression and activity of these activators in response to specific environmental signals (Buck et al., 2000; Reitzer and Schneider, 2001). The σ^{54}-dependent activators contain a regulatory domain that controls their ATPase activity (Buck et al., 2000; Shingler, 1996; Wedel and Kustu, 1995). A common mechanism used to control the interaction of the regulatory domain with the ATP-binding domain is phosphorylation, as illustrated by the nitrogen regulator activator protein NtrC. The NtrC family of activators is one of the best-studied examples of regulatory proteins that use DNA looping to activate transcription from σ^{54}-dependent promoters (Buck et al., 2000; Weiss et al., 1991).

Other activators, such as NifA (controls nitrogen fixation regulon) and PspF (controls phage shock protein regulon), do not use phosphorylation as

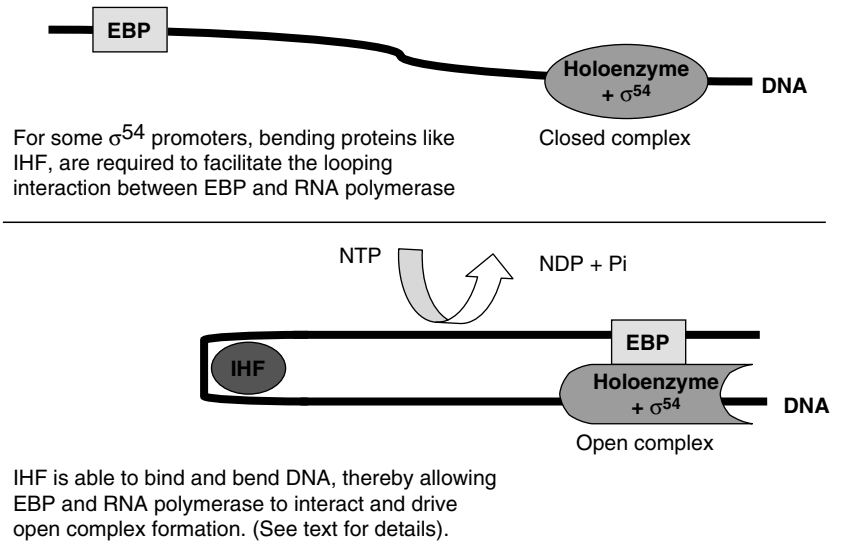
EBP
Holoenzyme
+ σ54
DNA
For some σ54 promoters, bending proteins like IHF, are required to facilitate the looping interaction between EBP and RNA polymerase
Closed complex
NTP
NDP + Pi
EBP
IHF
Holoenzyme
+ σ54
DNA
Open complex
IHF is able to bind and bend DNA, thereby allowing EBP and RNA polymerase to interact and drive open complex formation. (See text for details).

FIGURE 13. Transcription initiation by σ^{54}-containing RNA polymerase. See text for additional details.

a mechanism of activating transcription from σ^{54} promoters, but rather interact with small-molecule effectors or inhibitory peptides (Dixon, 1998; Dworkin et al., 2000; Shingler, 1996; Xiao et al., 1994). Regardless of the mechanism of activation of σ^{54} promoters the following is true: when the appropriate environmental stimulus is present, the ATPase activity of the activator is triggered by a conformational change mediated by one of the regulatory mechanisms described above, whereas when the stimulus is removed, the ATPase activity is silenced and the cognate σ^{54} promoters revert to the inactive closed complex conformation.

2.6.1. Role of σ^{54} in Bacterial Pathogenesis

Sigma 54 plays an important role in the virulence and pathogenesis of a variety of bacterial pathogens, including *Pseudomonas* spp., *Vibrio* spp., *B. burgdorferi*, *P. mirabilis*, and a variety of enteric bacteria, including *Salmonella* (Studholme and Buck, 2000). In *P. aeruginosa*, σ^{54} is important for virulence in several infection models, including infection of the respiratory epithelium in cystic fibrosis xenografts, in a mouse thermal or burn injury model, and in the nematode *Caenorhabditis elegans* (Cohn et al., 2001; Hendrickson et al., 2001). The reduced virulence of *P. aeruginosa* strains carrying loss-of-function mutations in *rpoN* (encodes σ^{54}) may be due, in part, to the fact that σ^{54} mediates the expression of pili and flagella, two important adherence factors (Ishimoto and Lory, 1989; Mahenthiralingam et al., 1994; Totten et al., 1990). However, given the pleiotropic effects of *rpoN* mutations

in *P. aeruginosa*, it is likely that σ^{54} regulates the expression of other virulence factors as well (Mahenthiralingam et al., 1994). In this regard, σ^{54} has also been shown in *P. aeruginosa* to regulate quorum sensing (a type of intercellular communication used by bacteria to coordinate group behavior) and biofilm formation (Thompson et al., 2003). In *V. cholerae*, σ^{54} facilitates colonization of the mouse intestinal tract following infection and controls flagellar biosynthesis (Klose and Mekalanos, 1998). In *B. burgdorferi*, σ^{54} controls the expression of RpoS, which regulates the expression of key *Borrelia* outer-membrane lipoproteins, including OspC and DbpA. These proteins may play roles in the pathogenesis of Lyme disease, as evidence suggests that OspC may serve as an immune target and DbpA as an adhesin to host tissues (Guo et al., 1998; Liang et al., 2002; Scheiblhofer et al., 2003; Wilske et al., 1993). Thus, the RpoN–RpoS regulatory network in *B. burgdorferi* may facilitate the organism's parasitic lifestyle and ability to cause disease (Hubner et al., 2001). In addition, RpoN-regulated genes have also been implicated in the pathogenesis of urinary tract infections caused by *P. mirabilis* (Zhao et al., 1999). It is also interesting to note that σ^{54} may play a role in bacterial virulence via its regulation of members of the phage shock protein (*psp*) operon. Transcription of the *psp* operon is absolutely dependent upon σ^{54} and its interaction with the transcriptional activator protein PspF (Jovanovic et al., 1996; Model et al., 1997). The *psp* locus appears to help the bacterial cell adapt to changes in membrane integrity and is known to be strongly induced by a family of proteins called secretins that function to assist export of large complexes from the cell, including virulence factors exported by T2SS and T3SS (Brissette et al., 1991; Model et al., 1997; Russel, 1994; Weiner et al., 1991). The *psp* operon and its associated secretins have been implicated in bacterial virulence. For example, the *psp* locus of the gastrointestinal pathogen *Y. enterocolitica* is required for virulence, by aiding efficient protein translocation through T3SS (Darwin and Miller, 2001). Additional studies of the *psp* operon in other bacterial pathogens have shown that the *E. coli pspA* operon is induced during biofilm formation and the *S. enterica pspA* operon is induced during macrophage infection (Beloin et al., 2004; Eriksson et al., 2003).

3. Conclusions

Bacterial alternative sigma factors are key global adaptive response regulators that play important roles in bacterial stress resistance and pathogenesis. During the natural course of their life cycles, bacterial pathogens encounter a wide range of stresses, both in the environment and in the infected host. To survive these stresses, a pathogen must be able to adapt quickly to what are often dramatically different environments. A common method of adaptation to stress in bacteria is to switch the sigma factor used by the core RNA polymerase. This reversible binding of alternative sigma factors to core RNA

polymerase allows formation of different holoenzymes that are able to distinguish groups of promoters required for different cellular functions. This results in the coordinated regulation of expression of specialized gene sets whose products are required for survival in the new environment. Bacteria have a principal sigma factor that is responsible for the transcription of essential housekeeping genes and a variable number of alternative sigma factors that are transcriptionally and/or posttranslationally activated in response to specific environmental signals. Some alternative sigmas provide bacteria with a general stress response that confers cross-protection against multiple stresses, while others confer specific stress resistance upon the cell. The majority of alternative sigmas belong to a single family of proteins, which are structurally and functionally related to the major *E. coli* sigma factor, σ^{70} (σ^{A}). An exception is the sigma factor σ^{54} (σ^{N}), which represents a second family of sigmas that is widely distributed in prokaryotes.

Questions to Consider

1. How do sigma factors regulate gene expression?

Sigma factors reversibly bind to the core RNA polymerase to form the holoenzyme, which is then competent to recognize and bind to a specific class of promoters. The formation of the RNA polymerase holoenzyme is a crucial step in the initiation of transcription, as neither the core RNA polymerase (i.e., without sigma) nor the sigma subunit alone are able to specifically recognize and bind to the promoter sites that signal the starts of genes. Thus, sigma confers promoter-specific transcription initiation on RNA polymerase and, as such, plays a major role in gene expression.

2. What is the difference between a primary sigma factor and an alternative sigma factor?

The primary sigma factor is the major sigma that is responsible for directing general transcription and the bulk of RNA during exponential growth of bacteria (housekeeping genes). It is essential for bacterial growth under normal physiological conditions. The primary sigma is reversibly associated with RNA polymerase and can be replaced by alternative sigma factors. Alternative sigma factors possess different promoter recognition properties compared to the primary sigma, and regulate the expression of genes that are important for bacterial survival during suboptimal or stressful growth conditions. In general, alternative sigmas are not essential for bacterial growth under normal physiological conditions. Most bacteria have a single primary sigma factor, but have a variable number of alternative sigmas.

3. How do sigma factors belonging to the σ^{70} family differ from the σ^{54} family?

Members of the σ^{54} family share no sequence homology with the σ^{70} family, and direct the RNA polymerase to promoter sequences that are much different than those that are important for recognition by the σ^{70} family. The σ^{54}-

containing holoenzyme recognizes a pair of promoter elements that are located at −12 and −24 (compared to −10 and −35 for σ^{70} promoters) that have the consensus sequences TGGCAC and TTGCA/T, respectively (compared to TTGACA and TATAAT for the −10 and −35 regions in σ^{70} promoters). In addition, σ^{54} family members also use a distinct pathway of open complex formation compared to σ^{70} family members. Whereas for all σ^{70} family members the process of open complex formation is driven entirely by using free energy from binding and requires no coupled hydrolysis of nucleotides or other free energy inputs, open complex formation by the σ^{54} family requires nucleotide hydrolysis that is driven by interaction with a specialized bacterial enhancer-binding protein (EBP).

4. How is σ^{38} regulated at the translational and posttranslational levels?

Sigma 38 is regulated at the translational level by control of rpoS mRNA secondary structure. During periods of normal physiological growth, rpoS mRNA is folded into a structure that "hides" the ribosomal binding site, and thus translation is impeded. However, in response to different environmental stresses, translation of *rpoS* is thought to be stimulated by rearrangements of *rpoS* mRNA secondary structure that render the ribosomal binding site accessible to the protein-synthesizing machinery. Several proteins and small regulatory RNAs regulate translation by affecting the secondary structure of *rpoS* mRNA. Levels of RpoS are further regulated by posttranslational alterations in protein stability through the action of proteases. The molecular components regulating RpoS stability include the ClpXP protease and the response regulator protein RssB, which is required for targeting σ^{38} to the ClpXP protease for degradation. Regulation of the phosphorylation state of RssB is essential in determining the turnover rate (and hence stability) of σ^{38}. Specifically, under normal physiological growth conditions (i.e., nonstress), RssB exists in a phosphorylated form, which has a high affinity for binding σ^{38}. Binding of RssB to σ^{38} facilitates transfer of σ^{38} to ClpXP, and its subsequent degradation. This is why levels of σ^{38} are low in exponentially growing cells. However, during times of stress, RssB is believed to exist in a dephosphorylated state, in which it has reduced affinity for binding σ^{38}, thus increasing σ^{38} stability.

5. What role do chaperones and proteases play in the σ^{32} and σ^{54}-mediated heat shock response?

In response to a sudden increase in temperature, bacteria induce the expression of HSPs that are designed to promote survival of the cell at elevated temperatures. The main strategy of the bacterial heat shock response is defense from denatured (i.e., unfolded and misfolded) proteins, which accumulate during thermal stress. Accordingly, the bacterial HSPs are designed to repair damage to proteins caused by thermal stress. Many bacterial HSPs are molecular chaperones or proteases that function by facilitating refolding of damaged proteins or degrading proteins that cannot be repaired. This adaptive

response is essential for cell survival during thermal stress, since denatured proteins are unable to perform their specific cellular functions. Since cellular survival depends on maintaining proteins in their proper functional conformations, the cell carefully regulates the expression of chaperones and proteases to compensate for heat shock, as well as other stresses that cause abnormal protein folding, including exposure to ethanol, heavy metals, DNA-damaging agents, antibiotics, oxidative stress, and phage infection.

6. How do antisigma factors regulate the activity of sigmas?

Antisigma factors act as antagonists to sigma factors and play an important role in controlling the activity of their respective sigmas in response to environmental cues. An antisigma factor is defined by its ability to inhibit transcription from a given promoter by inhibiting only the sigma factor that is required to recognize that specific promoter sequence.

7. What is the major difference in the types of stress responses mediated by σ^{38} and σ^{B} compared to the other alternative sigma factors discussed in this chapter?

Sigma 38 and σ^{B} are general stress response sigmas that provide general and rather nonspecific protection under a variety of adverse conditions. This adaptive response is particularly important for cells in a growth-restricted state, and serves to equip them with multiple stress resistance in preparation for surviving future stresses. This multiple stress resistance is in stark contrast to that mediated by specific stress responses, which are activated by a single stress signal and control the expression of proteins that provide protection against that specific stress only. While specific stress responses act to eliminate the offending stress and/or repair cellular damage that has already occurred, the general stress response renders cells broadly resistant such that damage is avoided rather than requiring repair. Thus, by providing cross-protection against multiple stresses, the major function of the general stress response is preventative in nature.

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Chapter 13

Two-Component Regulatory Systems

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Historical Landmarks

1960 Expression of several virulence determinants of *Bordetella pertussis* was altered by changing growth conditions, a phenomenon now referred to as "phenotypic modulation" (Lacey, 1960).

1969 Mutations in *crp* or *cya* of *Escherichia coli* resulted in the same phenotype: the inability to grow on many carbon sources other than glucose (Perlman and Patan, 1969; Zubay et al., 1970).

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1985 Amino acid sequence of the *E. coli* chemotaxis protein CheY was shown to be related to other regulatory proteins (Stock et al., 1985).

1986 Two-component regulatory system (TCR) for nitrogen assimilation described (Nixon et al., 1986).

1988 The chemotaxis histidine kinase CheA and the nitrogen regulator NR_{II} of *E. coli* are shown to catalyze the autotransfer of the γ-phosphate of ATP to one of their histidine residues (Ninfa et al., 1988).

1988 The *E. coli* nitrogen regulator NR_{II} is shown to transfer the phosphate of NR_{II}-phosphate to an aspartate of NR_I (Weiss and Magasanik, 1988).

1989 Comparisons of central and carboxyl-terminal portions of response regulators reveal that helix-turn-helix motifs (DNA binding) are conserved across many of these proteins (Albright et al., 1989; Stock et al., 1989b).

1992 Bacterial response regulators can be phosphorylated by low–molecular weight phosphodonors (Feng et al., 1992; Lukat et al., 1992).

2003 Phenotypic microarray analysis of *E. coli* K-12 mutants with deletion of all TCRs (Zhou et al., 2003).

1. Introduction

Bacterial cells must sense and respond to environmental conditions that require signal transduction across their cell membranes. One of the most common mechanisms of transmembrane signal transduction in bacteria is through the utilization of two-component regulatory systems (TCRs) composed of two proteins: (1) a histidine kinase (HK) and (2) a response regulator that are usually encoded by contiguous genes on the chromosomes of most bacteria. A majority of these systems combine signal recognition, signal transduction, and gene activation in a two-protein system. The two-protein components utilize phosphorylation as a means of information transfer between each other. TCRs exist in many different configurations that range from the simple layout depicted in Figure 1A to multiple phosphorelay schemes and more than two proteins (Figure 1 B). Most of these phosphorelay systems include hybrid kinases in which both the His- and Asp-containing motifs are present within the same protein. Some of these systems contain an auxillary His-containing phosphotransferase (HPt) protein that transfers the phosphoryl message to the response regulator. The sensor kinase is the primary signal transduction protein that interacts directly with a signal ligand or with a receptor that binds to the signal ligand. Binding of the ligand induces an autophosphorylation reaction in which the γ-phosphate of ATP is transferred to a histidine residue on the kinase. The signal information now exists as a phosphoryl moiety usually attached to a conserved histidine

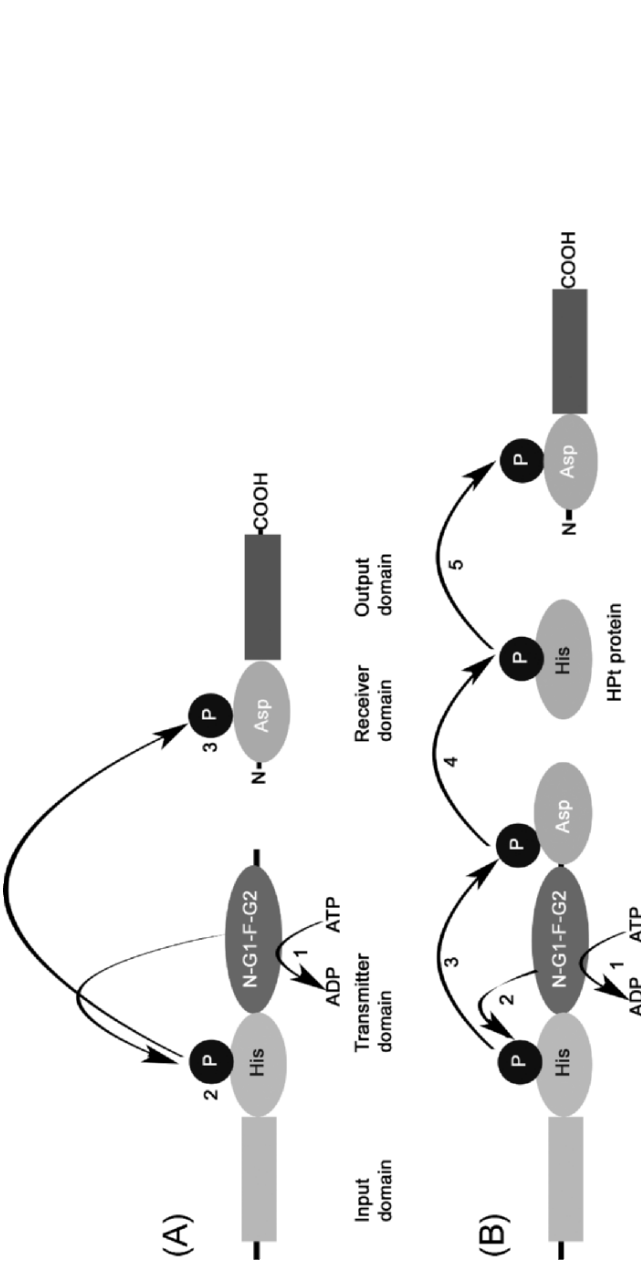

FIGURE 1. Schematic diagram depicting the modular organization of two-component regulatory systems (TCRs). (A) A prototypical TCR is composed of a histidine kinase (HK) (on left) comprising an input domain (light blue), and a transmitter domain (yellow and purple). The transmitter domain can be subdivided into a His-containing domain (yellow) and an ATP-binding domain (purple). The ATP-binding domain contains conserved N, G1, F, and G2 boxes (see text for description). The TCR is composed of an Asp-containing receiver domain (green) and a variable output domain (dark blue). Numbered arrows represent the path of the γ-phosphate of ATP that is transferred between the HK and the TCR. (B) Schematic diagram depicting an unorthodox HK involved in phosphotransfer network. The HK contains an Asp-receiver domain that passes its phosphate to a histidine phosphotransfer (HPt) protein, which ultimately phosphorylates the Asp of the TCR. There are a number of other arrangements for modules of TCRs (see text for details). (See color insert.)

residue to be transferred to a conserved aspartate on the response regulator. Each sensor kinase is mated to a response regulator protein that carries out the action, usually activation of specific gene transcription, to respond to the signal. In general, response regulator transcription factors consist of two major domains, the receiver or response domain and a DNA-binding domain, although there are numerous exceptions to this configuration (see below). Phosphorylation of the response regulator domain activates the transcription-regulating functions of the DNA-binding domain. The genes that this protein controls are determined by the specificity of the DNA-binding domain. Response regulators are the on–off switch in this system dependent upon their state of phosphorylation. Hundreds of TCRs have been identified in eubacteria, archaea (Ashby, 2004), and a few eukaryotic organisms (Li et al., 2002; Maeda et al., 1994; Srikantha et al., 1998; Wurgler-Murphy and Saito, 1997).

Pathogenesis (Bader et al., 2003; Cotter and Miller, 1997; Derzelle et al., 2004; Garcia Vescovi et al., 1994; Groisman et al., 1989; Gunn et al., 1996; Lizewski et al., 2002; McGowan et al., 2002; Miller et al., 1989; Oyston et al., 2000; Panthel et al., 2003; Perez et al., 2001; Pollack et al., 1986; Scarlato et al., 1991; Stephenson and Hoch, 2002, 2004), starvation for various nutrients (Antelmann et al., 2000; Moncrief and Maguire, 1998; Mueller et al., 1992; Ochsner and Vasil, 1996; Shi and Hulett, 1999; Song et al., 2004; Waldburger and Sauer, 1996), and responses to differing oxygen tensions (Agron et al., 1993; de Philip et al., 1990; Dixon, 1998; Jones and Haselkorn, 1989; Kadioglu et al., 2003; Lissenden et al., 2000; Miyatake et al., 1999; Oh and Kaplan, 2000; Rickman et al., 2004; Roberts et al., 2004; Saito et al., 2003; Sherman et al., 2001; Unden et al., 1994, 1995; Yarwood et al., 2001) are but a few of the changes in cellular physiology that are mediated by TCRs. A recent survey of the number of two-component response regulator genes in the complete and annotated genome sequences of prokaryotes (123 in June 2003) has revealed that there is a direct relationship between the number of potential protein-coding genes in a bacterium and the average number of response regulator genes. However, pathogens have the lowest average number of response regulators, followed by enterics with organisms from soil and water having a high average number of response regulators. Organisms that have complex lifestyles (e.g., form cysts, or symbiotic) have the highest average number of response regulators (Ashby, 2004).

There are numerous reviews of the biochemistry, structure, and functions of TCRs (Parkinson and Kofoid, 1992; Stock et al., 1989b; West and Stock, 2001) including one monograph (Hoch and Silhavy, 1995) to which the reader is referred for greater detail.

2. Structure and Function of Histidine Kinases

Members of the HK superfamily range in size from <40 kDa to >200 kDa with the vast majority of sensors being membrane-bound, homodimeric proteins (Grebe and Stock, 1999; West and Stock, 2001). The sensor kinases

are modular in architecture and can be roughly broken into four domains, moving from the amino-terminus to the carboxyl-terminus: (1) sensing domain; (2) linker domain; (3) phosphotransferase domain; and (4) the kinase catalytic core (see below). The amino-terminal sensing domain of sensor kinases is associated with the periplasm and attached to a cytoplasmic signal transduction (kinase) domain. In general, the carboxyl terminus of the sensor kinase is involved in the transduction of the phosphate "message" to the response regulator. The carboxyl terminus is also involved in the generation of the "message" by autophosphorylation. The sensor kinases also contain an ATP- or GMP-binding site from which the γ-phosphate of the nucleotide is removed. In general, the transfer of phosphate from the nucleotide results in the phosphorylation of a conserved histidine residue within the sensor kinase, hence the term HK (Figure 1A). This autophosphorylation reaction occurs between homodimers whereby one HK monomer catalyzes the phosphorylation of the conserved histidine residue in the second monomer (Stock et al., 2000). In addition, many HKs have phosphatase activity allowing them to dephosphorylate their target response regulators (Stock et al., 2000). HKs can be broadly classified into orthodox and unorthodox or hybrid kinases (Parkinson and Kofoid, 1992). Recently, the HK superfamily has been organized into subfamilies based upon homologies taken from sequenced prokaryotic genomes (Grebe and Stock, 1999; Kim and Forst, 2001). Approximately 20% of all HKs are hybrid or unorthodox kinases that contain additional domains. These domains may be either response regulator domains (see below) or histidine phosphotransfer (HPt) domains that undergo a "phosphorelay" (Figure 1B; Grebe and Stock, 1999).

2.1. Sensing Domains of Histidine Kinases

Usually, the N-terminal domain of HKs is responsible for detecting environmental stimuli either directly or indirectly. Due to the vast number of stimuli to which various HKs respond, there is little primary sequence similarity between different HKs. If the HK is found on the inner membrane (for Gram-negative organisms) or the outer membrane (for Gram-positive bacteria), this region of the HK will also contain hydrophobic regions that target these proteins to these surfaces. However, there are also cytosolic sensing modules that are integrated into HKs. One of the best studies of these is a family of HKs that contain a PAS domain (Taylor and Zhulin, 1999). The acronym PAS is derived from the names of three proteins in which these imperfect repeat sequences were first recognized: the *Drosophila* period lock protein (PER), vertebrate aryl hydrocarbon receptor nuclear translocator (ARNT), and *Drosophila* single-minded protein (SIM) (Nambu et al., 1991). PAS domains are found in all three kingdoms of life: *Bacteria*, *Archea*, and *Eucarya*. These domains are important for monitoring changes in light, redox potential, oxygen, small ligands, and

overall energy of the cell, and comprise a domain of approximately 100–120 amino acids (Taylor and Zhulin, 1999).

PAS domains have been identified to be important for the virulence of at least two important bacterial lung pathogens, *Mycobacterium tuberculosis* (Rickman et al., 2004) and *Streptococcus pneumoniae* (Echenique and Trombe, 2001; Wagner et al., 2002). *M. tuberculosis* contains 11 complete pairs of TCRs; one of the sensors encoded by *senX3* contains a PAS-like domain, and a strain harboring a mutation in this gene was avirulent in a murine tuberculosis infection model (Rickman et al., 2004). In the *S. pneumoniae vic* operon (*vicRKX*), VicK contains a PAS domain and has been shown to be essential for growth and competence in this organism (Wagner et al., 2002). Repression of competence in *S. pneumoniae* has also been shown to be controlled by another PAS domain containing kinase MicB (Echenique and Trombe, 2001).

2.2. Linker Domains of Histine Kinases

If the HK is located in the membrane, the sensing domain is connected to the cytoplasmic kinase portion of the HK through a transmembrane helix and a cytoplasmic linker. The linker regions of HKs are variable from 40 to 180 amino acids (Fabret et al., 1999). Multiple studies have indicated that this region is critical for signal transduction (Atkinson and Ninfa, 1992; Boyd, 2000; Collins et al., 1992; Fassler et al., 1997; Mattison et al., 2002; Park and Inouye, 1997). One recent study has indicated that the *Pseudomonas aeruginosa* kinase PilS, required for twitching motility, is located at the poles of the bacterium. Additionally, the linker and transmembrane domains of PilS are required for retention to the poles (Boyd, 2000). These regions are predicted to be composed of an α-helical coiled coil of approximately 50 amino acids. The coiled coils may be used as a structural relay, promote intramolecular associations, or correct structural alignment of monomers within the HK dimer (Stock et al., 2000).

2.3. Histidine Phosphotransfer Domains of Histidine Kinases

Histidine phosphotransfer (HPt) domains were initially identified in regulation of *Bacillus subtilis* sporulation where Spo0F was determined to be a second messenger for Spo0B, which transfers its phosphate to Spo0A (Burbulys et al., 1991). The HPt domains do not exhibit either kinase or phosphatase activity and serve as cross-communication proteins between various parts of a phosphotransfer network. HPt domains may either exist within HKs as in the *Escherichia coli* anoxic redox control protein ArcB (Unden et al., 1995) or as separate proteins as in the case of Spo0B in *B. subtilis* (Burbulys et al., 1991). The structures of four HPt domains have been determined: (1) *E. coli*

CheA (Zhou et al., 1995); (2) *E. coli* ArcB (Kato et al., 1997); (3) *B. subtilis* Spo0B (Varughese et al., 1998), and (4) *Sacchromyces cerevisiae* YPD1 (Xu and West, 1999). All of these HPt domains share a common four-helix motif despite their lack of sequence homology. Interestingly, the structure of these domains are homologous to the dimerization/histidine containing domain of EnvZ. One important HK that contains a HPt domain and is important for pathogenesis is the *B. pertussis* HK BvgS (discussed below) (Arico et al., 1989).

2.4. Kinase Catalytic Core of Histidine Kinases

The defining structural component of HKs is the kinase core composed of a dimerization region and an ATP/ADP-binding phosphotransfer domain (Grebe and Stock, 1999). Indications that two functional domains existed were evidenced by deletion analysis of the *E. coli* osmosensor EnvZ (Park et al., 1998). The crystal structure of the carboxyl-terminal portion of the *Thermotoga maritime* motility HK CheA protein, as well as subsequent crystallization of various HKs from different organisms, have confirmed these domains (Bilwes et al., 1999; Rajagopal and Moffat, 2003; Stewart, 2005). The kinase portion of HKs is more highly conserved compared to the amino-terminal-sensing domain, and can be identified by primary sequence motifs designated H, N, G1, F, and G2 boxes (Parkinson and Kofoid, 1992; Stock et al., 1989b). The H box contains a conserved histidine responsible for accepting the high-energy phosphate from the nucleotide-binding fold of an adjacent monomer of the kinase and transferring this phosphoryl group to the aspartate of the response regulator. In the majority of HKs, the H box is part of the dimerization domain but it may be found at the amino-terminal end of the HK within an HPt domain. The N, G1, F, and G2 domains contain a conserved asparagine (N), conserved glycines (G1 and G2), and a phenylalanines (F), which together function as an ATP-binding site (Parkinson and Kofoid, 1992; Stock et al., 1989b; Figure 2).

3. Structure and Function of Response Regulators

Similar to the sensor proteins, the response regulators of TCRs are modular in structure and function. In general, the amino-terminal portion of response regulators are termed "receiver" domains because these domains receive the phosphoryl group of sensors on a conserved aspartate residue. The carboxyl-terminal portion of the response regulator generally contains a DNA-binding domain that is specific for the recognition sequence of the response regulator.

In a growing number of cases transcription of specific genes is known to be governed by oxidation or reduction of electron carriers with which the gene products interact. The biological function of such control is to activate

FIGURE 2. Radicicol bound to the ATP-binding site of the N-terminal domain of the yeast Hsp90 (Prodromou et al., 1996, 1997; Roe et al., 1999) as modeled from the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=cdd) (Marchler-Bauer et al., 2005). The transmitter domains or catalytic cores of histidine kinases are composed of an ATP-binding site and a His-containing site. Yellow denotes the N-box, red denotes the F-box and G-box. These boxes form an ATP-binding pocket. Alpha-helices are depicted as magenta tubular arrows and β-sheets are shown as magenta flat arrows. (See color insert.)

synthesis of appropriate redox proteins, and repress synthesis of inappropriate ones, in response to altered availability of specific electron sources and sinks. In prokaryotic systems this control appears to operate by two general classes of mechanisms: by two-component regulation involving protein phosphorylation on histidine and aspartate; and by direct oxidation-reduction of gene repressors or activators. For the first class, termed two-component redox regulation, the term redox sensor is proposed for any electronic carrier that becomes phosphorylated upon oxidation or reduction

and thereby controls phosphorylation of specific response regulators, while the term redox response regulator is proposed for the corresponding sequence-specific DNA-binding protein that controls transcription as a result of its phosphorylation by one or more redox sensors. For the second class of redox regulatory mechanism, the term redox activator protein and redox repressor protein are proposed for single proteins containing both electron transfer and sequence-specific DNA-binding domains (Allen, 1993).

3.1. Phosphoryl-Aspartate Receiver Domains

The single domain response regulator for *E. coli* chemotaxis CheY is the best studied for response regulator receiver domains (Bellsolell et al., 1994, 1996; Halkides et al., 2000; Jiang et al., 1997; Moy et al., 1994; Santoro et al., 1995; Stock et al., 1993; 1989a; Usher et al., 1998; Volz and Matsumura, 1991; Volz et al., 1986; Zhu et al., 1997). These studies have established that CheY is composed of five central parallel β-sheets surrounded by five α-helices (Stock et al., 1989a; Volz and Matsumura, 1991). The site of phosphorylation within CheY is Asp57 (Volz and Matsumura, 1991). This site is surrounded by other acidic residues (Asp12 and Asp13), and two other highly conserved Thr87 and Lys109 surround the active site of the regulatory domain (Volz and Matsumura, 1991). This acidic cluster is involved in binding of Mg^{2+} that is required for phosphorylation and dephosphorylation of CheY (Lukat et al., 1990, 1991; Needham et al., 1993). The crystal structures of numerous other response regulators have also been determined including the *B. subtilis* sporulation factor Spo0F (Madhusudan et al., 1996), the *Salmonella typhimurium* nitrogen response regulator NtrC (Volkman et al., 1995), the *E. coli* phosphate regulator PhoB (Sola et al., 1999), the *E. coli* nitrate regulator NarL (Baikalov et al., 1996), the *E. coli* chemotaxis protein CheB (Djordjevic et al., 1998), and the *Sinorhizobium meliloti* nitrogen fixation regulator FixJ (Birck et al., 1999). The overall structural trends of the regulatory domains initially observed in CheY are also observed within these proteins. *S. meliloti* FixJ is illustrated in Figure 3 as an example of these structural conservations. Multisequence analysis of known receiver modules that have been biochemically or genetically associated with one or more HKs revealed that these domains fall into 11 basic categories based upon the first characterized domain of its kind. The 11 basic categories are: (1) ActR; (2) CheB; (3) tandem; (4) CitB; (5) ComE; (6) FixJ; (7) NtrC; (8) Null; (9) H-2 domain; (10) LytR; and (11) OmpR (Grebe and Stock, 1999). The main difference between these basic categories is in the type or lack of output domains (see below).

3.2. Output Domains of Response Regulators

Most response regulators contain a DNA-binding region that is readily identified by a helix-turn-helix motif. However, there are a few response regulators that contain enzymatic activity instead of the DNA-binding region. One

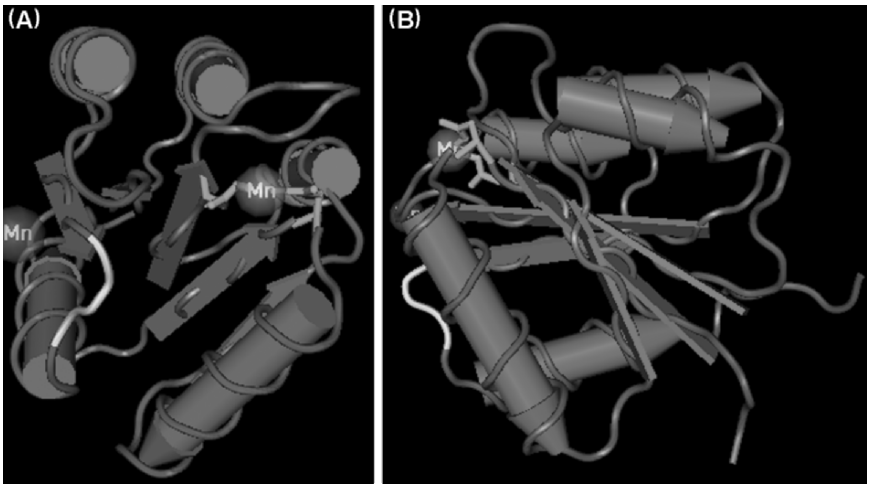

FIGURE 3. The nitrogen fixation response regulator FixJ of *Sinorhizobium meliloti* as modeled from the Conserved Domain Database (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?CMD=search&DB=cdd) (Marchler-Bauer et al., 2005). The representation of FixJ in A was rotated 90° to obtain B. The regulatory region of FixJ contains five α-helices (tubular arrows) surrounding five β-sheets (flat arrows). The side chains represent conserved aspartates that form an acidic pocket that binds Mn and forms the active site for phosphoryl transfer. The thin tube represents the dimerization domain of FixJ.

of the best-known examples is *E. coli* CheB of the flagellar chemotaxis system that contains a methylesterase (Stock and Koshland, 1978). Recently, response regulators that have alternative output domains (AAA-type, GGDEF, EAL, and HD-GYP domains) have also been identified (Galperin et al., 2001). Most of these motifs have been identified as a result of genomic comparison between over 200 bacterial genomes that are currently available in the databases. There are also response regulators that do not contain the conserved helix-turn-helix motif but contain other types of DNA-binding motifs such as a "winged helix" or a LytR-type (Nikolskaya and Galperin, 2002). OmpR and PhoB both contain a "winged helix" or "winged HTH" DNA-binding domain (Martinez-Hackert and Stock, 1997a, b). Examples of the LytR-type of DNA-binding domain include *P. aeruginosa* AlgR and *B. subtilis* LytR (Nikolskaya and Galperin, 2002). Approximately 25% of all response regulators have no output domain.

The output domain of response regulators is affected by the phosphorylation state of the receiver domain. Phosphorylation of the receiver domain likely induces a conformational change in the response regulator that either increases or decreases the affinity of the output domain for its cognate binding sequence. For response regulators that do not contain output domains,

it is believed that the affinity of the response regulator for its target effector protein(s) changes upon phosphorylation. For example, the affinity for CheY increases for the flagellar motor switch upon phosphorylation (Alon et al., 1998; Scharf et al., 1998; Welch et al., 1993).

4. Role Of Two-Component Regulators in Pathogenesis

Most pathogenic bacteria utilize virulence factors to establish and maintain infections, and many of these are controlled by TCRs. TCRs are required for the transcription of many different virulence factors including alginate and type-IV pili production in *P. aeruginosa*—AlgZ/R (Deretic et al., 1989; Yu et al., 1997), PilS/R (Hobbs et al., 1993)—and hemagglutinin production in *B. pertussis*—(BvgA/S (Roy and Falkow, 1991)—to name just a few.

The TCRs of many different pathogens have been identified through homologies to other known TCRs when the genomes of these organisms were sequenced. The first completed bacterial genome, *Haemophilus influenzae*, revealed that there were four two-component regulator/sensor pairs and one lone regulator (Fleischmann et al., 1995). The *arcAB* TCR has been characterized in *H. influenzae* and mediates signal transduction in response to redox conditions of growth despite the absence of a PAS domain (Georgellis et al., 2001). ArcA knockout mutations of a virulent type b strain of *H. influenzae* rendered this strain sensitive to human serum and greatly reduced virulence in BALB/c mice (De Souza-Hart et al., 2003). Insertional inactivation of other *H. influenzae* homologs of TCR genes had no effect on competence (Gwinn et al., 1996). The genome sequence of the respiratory pathogen *S. pneumoniae* revealed 13 TCRs that were systematically disrupted and tested in an intraperitoneal virulence model that resulted in no change in virulence for any of the mutant strains (Lange et al., 1999). However, only 11 of the 13 TCRs were disrupted in this study. The authors conclude that the other two TCRs are required for *S. pneumoniae* (Lange et al., 1999). Theses results were corroborated by an independent study; however, in contrast to the initial study, these authors found that 8 of the TCRs were attenuated in a murine respiratory tract infection model (Throup et al., 2000).

There are numerous other examples of TCRs that play a role in regulation of virulence. Instead of listing each individual case here, we will focus on two of the better-studied TCRs, PhoPQ and BvgAS, described below as examples.

4.1. The Gram-negative Two-Component Regulatory System PhoPQ

PhoP-PhoQ was first discovered in the intracellular pathogen, *S. enterica* serovar Typhimurium (Groisman et al., 1989; Kier et al., 1979; Miller et al., 1989). This two-component response regulatory system has been extensively

characterized at the molecular level for both *Salmonella* and non-pathogenic *E. coli*. PhoPQ appears to operate similarly in both bacteria, although the PhoPQ regulons of *E. coli* and *Salmonella* (as well as other Gram-negative pathogens) differ.

4.1.1. Identification and Characterization of PhoP-PhoQ in *Salmonella enterica* serovar Typhimurium and *Escherichia coli*

PhoP was first hypothesized as a regulator of the nonspecific acid phosphatase, PhoN, since chemically induced mutations in the *phoP* locus altered phosphatase activity in *S. enterica* serovar Typhimurium (Kier et al., 1979). The discovery that *phoP* transposon mutants, but not *phoN* mutants, were highly attenuated for virulence in vivo suggested that PhoP regulated the genes necessary for *S. typhimurium* pathogenesis (Fields et al., 1989). Shortly thereafter, two independent groups established that PhoP is a transcriptional regulator with homology to the previously characterized two-component response regulators, PhoB and OmpR (Groisman et al., 1989; Miller et al., 1989). A single gene, *psiD*, was identified to be PhoP-activated utilizing *lac* gene transcriptional fusions to phosphate starvation-inducible genes in strains harboring a wild-type *phoP* allele or a *phoP*:Tn10 mutation (Groisman et al., 1989). In addition to proving that PhoP could regulate unlinked loci, the authors demonstrated by Southern hybridization that homologs to *phoP* were present in numerous Gram-negative bacteria including the intracellular pathogens *Shigella flexneri*, *Yersinia pestis*, and *Neisseria gonorrhoeae*. They also identified a hexanucleotide repeat (GTTTAT) upstream of the putative transcriptional start site of *phoP* that would later be characterized as the PhoP box (see below) (Groisman et al., 1989).

An independent group sequenced the *phoP* regulatory locus as well and identified a second component, PhoQ (Miller et al., 1989). Analagous to that observed for PhoP, PhoQ showed high sequence similarity to the sensor kinase, CheA, of two-component response regulatory systems. In addition, the predicted transmembrane topology of PhoQ was similar to that of another sensor kinase, EnvZ. Since *phoP* and *phoQ* were positioned in the same orientation and overlapped by a single base pair, this suggested that they composed a single transcriptional unit. Mutations in either *phoP* or *phoQ* caused a 10,000-fold reduction in virulence of *Salmonella* as measured by the LD_{50} in the murine model of infection (Miller et al., 1989). This provided further evidence that PhoP-PhoQ functioned as a TCR. Three years after PhoP-PhoQ was identified in *Salmonella*, the *phoP-phoQ* operon in *E. coli* was cloned and sequenced (Kasahara et al., 1992). The amino acid sequences of PhoP and PhoQ of *E. coli* are 93% and 86% identical, respectively, to those of *S. typhimurium*. PhoP is 223 and 224 amino acids with a predicted molecular weight of 25.5 and 25.6 kDa in *E. coli* and *S. typhimurium*, respectively. PhoQ is 486 amino acids with a predicted mass of 55.2 KDa in *E. coli* and 487 amino acids with a predicted molecular

weight of 55.3 kDa in *S. typhimurium*. As observed in *S. typhimurium*, PhoP and PhoQ in *E. coli* are translationally coupled by the 1bp overlap of the stop codon of PhoP and the start codon of PhoQ. A PhoP box is also observed upstream of the transcriptional start site of *phoPQ* in *E. coli*. In both *E. coli* and *S. typhimurium*, a –10 consensus region, but not a –35 consensus region, is apparent in the promoter region of *phoPQ* (Groisman et al., 1992).

The most comprehensive analysis of the PhoPQ regulon in *E. coli* was provided by Minagawa et al. (2003). Using DNA microarray, the authors identified 232 genes that were either directly or indirectly regulated by PhoP-PhoQ in response to extracellular Mg^{2+}. Of the identified genes 26 contained the PhoP box in their promoter regions. S1 nuclease assays verified 6 new genes (*hemL*, *nagA*, *rstAB*, *slyB*, *vboR*, and *yrbL*) under the control of PhoP in addition to *phoPQ*, *mgrB*, and *mgtA*, which had been previously reported (Kato, et al., 1999; Minagawa et al., 2003). DNase I footprinting experiments confirmed that PhoP recognizes and binds to the PhoP box for each of these genes with the exception of *hemL* (Minagawa et al., 2003).

4.1.2. Regulation of the PhoP-PhoQ Operon

The PhoP-PhoQ operon was the first TCR shown to respond to an extracellular cation. An elegant study established that Mg^{2+} is the primary environmental signal for activation of the PhoPQ operon in *S. typhimurium* (Garcia Vescovi et al., 1996). Using an *S. typhimurium* PhoP-activated gene (*psiD*) fused to the lac reporter gene, the authors demonstrated that expression of *psiD* was inversely proportional to the Mg^{2+} concentration in the growth medium. Substitution of Mg^{2+} with Ca^{2+} or Mn^{2+} induced PhoPQ to some extent; however, other cations such as Ni^{2+}, Cu^{2+}, and Ba^{2+} had no effect on PhoPQ signal transduction. S1 nuclease mapping experiments of the PhoPQ promoters in response to Mg^{2+} levels confirmed these observations. In addition to *psiD*, the authors demonstrated that the PhoP-activated genes included the magnesium transport genes, *mgtA* and *mgtCB*. Furthermore, PhoP and PhoQ mutants were unable to grow in low Mg^{2+} media. To confirm the ability of PhoQ to sense extracellular Mg^{2+}, the authors constructed a chimera consisting of the N-terminal (periplasmic) domain of EnvZ and the C-terminal (cytoplasmic) domain of PhoQ, and demonstrated that the periplasmic domain of PhoQ was necessary to mediate a repressing effect of elevated Mg^{2+} levels. Trypsin digestion experiments indicated that millimolar levels of Mg^{2+} stimulate a conformational change in the periplasmic domain of the sensor, PhoQ, that leads to the downregulation of PhoP-activated genes.

In both *E. coli* and *S. typhimurium*, the sensor domain of PhoQ contains a cluster of acidic residues. In *E. coli*, replacement of these residues with uncharged amino acids reduces the ability of PhoPQ to respond to changes in divalent cations (Waldburger and Sauer, 1996). However, *P. aeruginosa* PhoQ does not contain this acidic cluster but is still able to substitute for

E. coli's periplasmic domain (Lesley and Waldburger, 2001). This suggests an alternative or more complex mode of signal recognition by PhoQ. In support of this, four residues outside of this acidic cluster and conserved among the PhoQ proteins from several enteric bacteria were shown to mediate Mg^{2+} signaling in vivo (Chamnongpol et al., 2003). Interestingly, the binding of Ca^{2+} to PhoQ appears distinct from that of Mg^{2+} (Regelmann et al., 2002; Vescovi et al., 1997). Maximal repression of PhoP-regulated transcription was achieved when *Salmonella* was grown in both MgCl and CaCl compared to either cation alone, suggesting an additive effect. Furthermore, a constitutively active *phoP* mutant (*pho*-24) responds normally to Mg^{2+}, but has a lower affinity for Ca^{2+} compared to wild-type PhoQ (Vescovi et al., 1997).

4.1.3. Signal Propagation and Phosphorelay

The transduction events following the binding of Mg^{2+} to PhoQ demonstrated that PhoQ possesses an autokinase activity (Castelli et al., 2000). Incubation of purfied PhoQ with ^{32}P-labeled ATP resulted in autophosphorylation of PhoQ in His277 (the predicted conserved histidine residue). Autophosphorylation was not observed when Val was substituted for His at residue 277. Next, PhoQ that had been autophosphorylated was purified and incubated with PhoP. Phosphorylation of PhoP was observed at the conserved aspartate residue 55 (Asp51 in *E. coli*). The rate of phosphotransfer increased with increasing amounts of Mg^{2+}, until the Mg^{2+} concentration reached 250 μM. At this point, phospho-PhoP became dephosphorylated, while phospho-PhoQ remained stable. The authors demonstrated that two independent mechanisms account for the dephosphorylation of PhoP. First, the phosphotransfer from PhoQ to PhoP was reversed in low divalent cation concentrations. Incubation of phospho-PhoP with PhoQ resulted in phosphorylation of PhoQ at His277. Moreover, upon the addition of ADP to phospho-PhoQ, PhoQ transferred its phosphate to ADP demonstrating a reversal of its autokinase activity as well. Second, and most important, PhoQ possesses phosphatase activity that is activated by Mg^{2+} binding to the N-terminal domain of PhoQ and resulted in the dephosphorylation of phospho-PhoP. When phospho-PhoP was incubated with PhoQ in the presence of millimolar amounts of Mg^{2+}, PhoP was dephosphorylated. The authors ruled out the possibility that PhoP possessed an autophosphatase activity based on the kinetics of the phosphate loss (Castelli et al., 2000).

Additional studies of point mutations in the putative PhoP phosphorylation site (Asp51 *E. coli*; Asp55 in *S. typhimurium*) and the PhoQ autophosphorylation site (His277) have confirmed that these residues are essential for PhoPQ signaling in response to low Mg^{2+} and subsequent activation of PhoP-regulated genes (Castelli et al., 2000; Kato et al., 1999). Furthermore, the His-phosphotransfer domain was essential for PhoQ's recognition and interaction with PhoP and so that this domain dimerizes (Castelli et al., 2003).

Specific mutations in the periplasmic domain of PhoQ may also alter its state of activity by causing a conformational change that switches PhoQ between the kinase- and phosphatase-dominant states (Regelmann et al., 2002; Sanowar et al., 2003). For example, a constitutively active *phoP* mutant (*pho*-24), which resulted in elevated acid phosphatase activity in *S. enterica* serovar Typhimurium, was later characterized as a threonine to isoleucine subsititution at residue 48 in the periplasmic sensor domain of PhoQ (Gunn et al., 1996). While the rate of autophosphorylation of PhoQ in the *pho*-24 mutation is the same as wild type, the *pho*-24 mutation results in lower phosphatase activity for PhoQ, and thus PhoP is constitutively active (Sanowar et al., 2003). Therefore, this residue appears important for PhoQ's ability to switch from a kinase to a phosphatase and to regulate the phosphorylation status of PhoP (Regelmann et al., 2002; Sanowar et al., 2003). Therefore, the kinase, phosphotransfer, and phosphatase activities of PhoQ each contribute to the regulation of PhoP by regulating the phosphorylation status of PhoP.

Interestingly, when overexpressed (albeit at nonphysiological levels) on a plasmid, PhoP is able to activate a target gene independently of its phosphorylation status (Lejona et al., 2004). This appears to be due to protein–protein interactions that are favored at high concentrations of PhoP. Both PhoP and phosphorylation-defective PhoP are able to form dimers as demonstrated by cross-linking experiments. However, dimerization is enhanced through phosphorylation of PhoP similarly to that observed for the response regulators PhoB, NtrC, NarL, and ArcA. Furthermore, the unphosphorylated form of PhoP cannot compete with phospho-PhoP since transcription of the *phoPQ* promoter remained unchanged upon induction of PhoP from a plasmid in a *phoQ* mutant (Soncini et al., 1995).

In summary, under Mg^{2+}-limiting conditions, the kinase activity of PhoQ predominates, resulting in the phosphorylation of PhoP, promoting its dimerization and the upregulation of PhoP-activated genes. Under Mg^{2+}-replete conditions, binding of Mg^{2+} to PhoQ stimulates a conformational change in its periplasmic domain and activates its phosphatase activity. This leads to the dephosphorylation of PhoP and the downregulation of PhoP-activated genes.

4.1.4. Transcriptional Regulation of PhoPQ- and PhoP-regulated Loci

Two different transcripts are produced from the *phoPQ* operon during growth of *S. typhimurium* in Mg^{2+}-limiting conditions: phoPp1, which requires both PhoP and PhoQ for activity and which is environmentally regulated; and phoPp2, which is constitutively active (Soncini et al., 1995). Transcription from PhoPp1 requires phosphorylated PhoP because its transcript is absent in *phoP* or *phoQ* mutants. In contrast, phoPp2 is active in the absence of PhoP or PhoQ and provides a basal level of expression of PhoP. Therefore, *phoPQ* positively autoregulated, in that low-level expression of PhoP from phoPp2 provides a source of PhoP for phosphorylation by PhoQ

in response to low Mg^{2+}. This phospho-PhoP can then bind phoPp1 to activate *phoPQ* transcription (Figure 4).

Two transcipts were also observed for *phoPQ* in *E. coli*. As with *S. typhimurium phoPQ*, phoPp1 was dependent on extracellular Mg^{2+} and was decreased in *phoP* or *phoQ* mutants, while phoPp2 was constitutively expressed in the presence or absence of Mg^{2+} (Kato et al., 1999).

As mentioned above, a direct repeat GTTTAT located 12–25 bp upstream of phoPp1 in both *E. coli* and *S. typhimurium* was reminiscent of DNA motifs recognized by other TCRs, such as PhoB and the PhoB box. However, the partial or complete absence of this motif in many PhoP-activated genes delayed DNase footprinting analyses. Finally, site-directed mutagenesis and DNase I footprinting confirmed that PhoP bound the heptanucleotide direct repeat TGTTTAA in *E. coli* (Yamamoto et al., 2002) and suggested that PhoP dimers could bind along the PhoP box in the same direction to initiate transcription. Presently, the PhoP box is only well characterized for *E. coli* and *S. typhimurium*. In *P. aeruginosa*, the presence of four hexanucleotide GTTCAG repeats upstream of the OprH-PhoP-PhoQ operon may represent a PhoP consensus motif (Macfarlane et al., 1999). However, DNase I footprinting analyses have not been performed for PhoP in *P. aeruginosa* to date.

In *E. coli* and *S. typhimurium*, the direct repeat (T/G) GTTTA was also observed upstream of the magnesium transport genes, *mgtA* and *mgtCB*, supporting its role in PhoP binding in response to low Mg^{2+} in the environment (Kato et al., 1999; Lejona et al., 2003; Soncini et al., 1996). However,

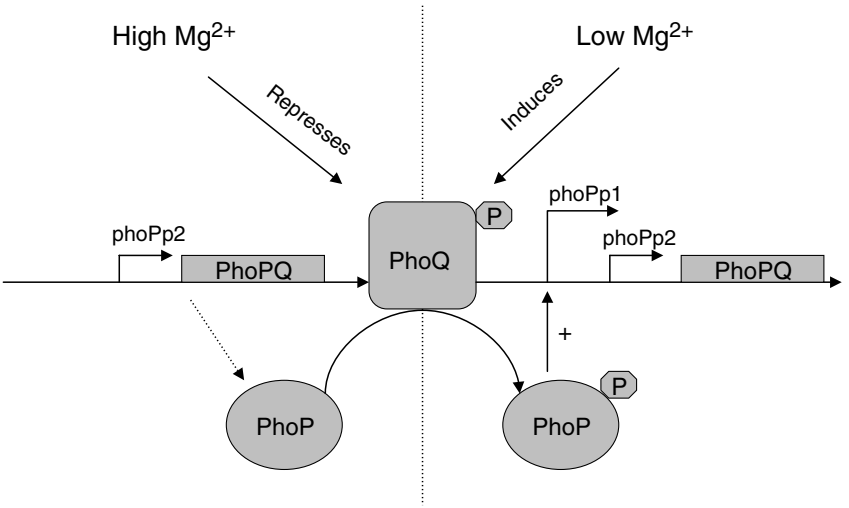

FIGURE 4. Model proposed by Soncini et al. (1995) for the autoregulation of *phoPQ* transcription in *Salmonella*. The constitutive promoter phoPp2 provides a basal level of PhoP expression. Upon induction, PhoP is phosphorylated and activates transcription via phoPp1, which upregulates PhoPQ.

a large number of PhoP-regulated loci lack this consensus motif. Therefore, the PhoPQ regulon appears to be both directly and indirectly regulated by PhoP in all bacterial species examined thus far. Each of these will be discussed below.

4.1.5. *Salmonella* PhoPQ Regulon

The PhoPQ regulon of *Salmonella* has received considerable attention over the last 15 years. Surprisingly, a DNA microarray–based study of PhoP-regulated loci in this pathogenic bacterium has not been published to date. Nonetheless, investigation of this TCR in *Salmonella* has led to the identification of key virulence genes, has shed light on the pathogenesis of *Salmonella*, and has led to the pursuit of *phoP* mutants as vaccine candidates.

Several PhoP-regulated loci appear to be common to the Enterobactereciae, such as the *phoPQ* operon, *mgtA*, and *slyB*. However, PhoP also regulates genes that are specific to *Salmonella* such as the *mgtCB* operon, *pagC* and *pagD*, *phoN*, and *mig14* (discussed below). The PhoP box is presently upstream of *phoPQ*, *mgtA*, and *slyB*, and DNase I footprinting confirmed that PhoP binds the PhoP box upstream of *phoPQ*. However, *mgtC*, *pagC*, *pagD*, and other virulence loci do not contain the PhoP box, suggesting that these loci are indirectly controlled by PhoP through another PhoP-regulated transcription factor (Lejona et al., 2003).

Indeed it appears that *Salmonella* has recruited PhoPQ to respond not only to changes in Mg^{2+} but to other environmental cues as well. A library of 50,000 *lac* gene transcriptional fusions recovered 47 genes that were activated by PhoP and 7 that were repressed by PhoP (Soncini et al., 1996). All but one of the PhoP-activated genes were induced under Mg^{2+}-limiting conditions and were not induced in low Mg^{2+} in *phoP* mutants. However, of the 25 PhoP-activated genes that were regulated by Mg^{2+} levels in the growth media, only 8 were required for growth in low-Mg^{2+} media. In addition, none of the 25 identified genes are known to play a role in calcium homeostasis (Soncini et al., 1996). This suggests that while PhoP governs the adaptation to low Mg^{2+}, it may also coordinate the response to other stresses, such as those encountered during infection of its host. For example, the concentrations of Mg^{2+} and Ca^{2+} in serum are 0.7–1 mM and 1–1.3 mM (Brown, 1994; Reinhart, 1988). In contrast, the phagosomal concentration of Mg^{2+} and Ca^{2+} are estimated to be less than 100 μM (Garcia-del Portillo et al., 1992; Pollack et al., 1986). Thus, the ability of *Salmonella* to survive in the hostile environment of the host macrophage may rely on Mg^{2+} sensing by PhoP-PhoQ, which in turn upregulates intracellular survival loci. One of the earliest indications that PhoPQ regulated virulence in *Salmonella* was that mutants that could not respond to changes in extracellular cations (i.e., *pho*-24 mutation) were avirulent in vivo. This eventually led to the characterization of PhoP-regulated virulence genes involved in invasion and intracellular survival; type III secretion; resistance to stress, defensins, and antimicrobials; and pathogenicity in vivo.

Miller et al. (1989) first demonstrated that PhoP-PhoQ regulates the expression of genes necessary for *Salmonella* virulence in mice. Mice were injected intraperitoneally and LD_{50}s were determined. Mutations in either *phoP* or *phoQ* resulted in over a 10,000-fold reduction in virulence. In addition, *phoP* and *phoQ* mutants did not survive as well as their parental strains in murine bone marrow–derived macrophages. Subsequent to this discovery, numerous studies utilizing transposon mutagenesis identified additional PhoP-regulated loci that were important for *Salmonella* pathogenesis (Belden and Miller, 1994; Miller et al., 1992b).

PhoP was first implicated as a regulatory locus of intracellular survival of *Salmonella* during a molecular genetic analysis of Tn10 insertion mutants that could not survive in murine peritoneal macrophages in vitro (Fields et al., 1989). The inability of *phoP* mutants to survive inside macrophages correlated with an increased susceptibility to mammalian defensins. Defensins are cationic antimicrobial peptides produced in the granules of host phagocytic cells and intestinal Paneth cells. Miller et al. (1989) utilized transposon mutagenesis to identifiy PhoP-regulated loci and discovered three unlinked loci, which they termed *pagA*, *pagB*, and *pagC* for "PhoP-activated genes." While *pagA* and *pagB* mutants did not show a virulence defect, *pagC* encoded a virulence factor that contributed to intracellular survival. *PagC* encoded an outer-membrane protein with sequence similarity to OmpX of *Enterobacter*, Ail of *Yersinia*, and Lom of lambda phage. Interestingly, *pagC* mutants were as resistant to defensins as their wild-type counterparts, but both *phoP* and *phoQ* were essential for defensin resistance (Miller et al., 1990). This suggested that PagC contributed to intracellular survival through an unknown mechanism and that the PhoPQ-regulated product involved in resistance to defensins remained to be characterized. The identification of 13 additional *pag* genes also failed to identify the locus involved in resistance to the mammalian defensin, NP-1 (Belden and Miller, 1994).

It is now apparent that PhoP regulates a number of loci involved in antimicrobial peptide resistance and that different genes mediate resistance to different antimicrobial peptides (Bader et al., 2003; Groisman et al., 1992). These include genes involved in the modification of lipopolysaccharide (LPS) and the production of extracytoplasmic proteases, as well as other unknown mechanisms of protection.

Mg^{2+} stabilizes the bacterial outer membrane by interacting with the phosphate groups in the LPS, thus reducing the charge repulsion by anions. Thus PhoP may upregulate genes that can reduce these repulsive forces when Mg^{2+} is limiting. Alternatively, it may restructure the LPS in order to utilize Mg^{2+} during Mg^{2+} limitation (Groisman, 2001). PhoP-regulated genes include *pagP*, which catalyzes the addition of palmitate to the lipid A moiety of LPS and confers resistance to the synthetic peptide C18G; the *pbgPE* operon (also referred to as the *pmr* operon), which incorporates 4-aminoarabinose into lipid A and mediates resistance to polymyxin; and *ugd*, which also mediates resistance to polymyxin via the addition of 4-aminoarabinose into lipid A

(Groisman, 2001). Interestingly, PhoP-PhoQ regulates the antimicrobial resistance of *Salmonella* by both direct and indirect mechanisms. While PhoP directly regulates *pagP*, PhoP regulates the *pbgPE* and *ugd* loci by upregulating the PmrA-PmrB TCR, which recognizes extracellular iron (Gunn and Miller, 1996). In *Salmonella*, this is accomplished by PhoP binding to the transcriptional regulator, *pmrD*, which in turn upregulates PmrAB (Figure 5). The PrmD promoter contains a single copy of the hexanucleotide PhoP motif. DNase I footprinting confirmed that PhoP binds, and thus directly regulates, *pmrD* (Kato et al., 2003).

Using differential fluorescence induction to select for PhoP-activated gene expression inside host cells, Valdivia and Falkow (1997) identified 14 macrophage-inducible proteins (Mig) that were PhoP-regulated in *Salmonella*. Mig-14 was later shown to be essential for lethal infection and to mediate resistance to polymyxin and protegrin-1 (Valdivia et al., 2000). Interestingly, *mig*-14 mutants contained no detectable differences in their LPS structure compared to wild-type *Salmonella* (Brodsky et al., 2002). Thus, the precise mechanism of Mig-14 protection awaits further characterization.

In addition to regulating the modification of LPS structure, PhoP appears to upregulate proteases that are able to degrade antimicrobial peptides. For example, PgtE, when overexpressed to high levels, is able to cleave C18G (Guina et al., 2000). Thus, PhoP activates several virulence loci that enhances its survival during infection. Early studies of the constitutively active *phoP*

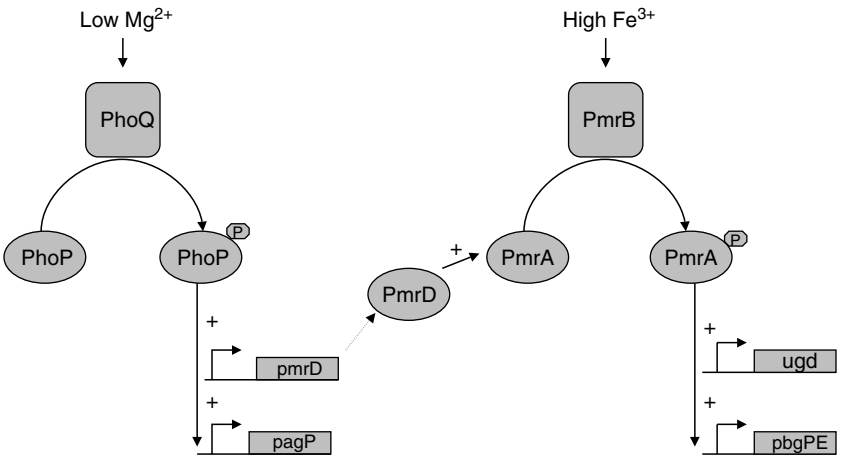

FIGURE 5. Schematic diagram of PhoP-regulated transcription of the LPS modification genes *pagP*, *ugd*, and *pbgPE* operon in *Salmonella*. PhoP directly regulates *pagP* transcription, while activation of *ugd* and *pbgPE* is mediated by transcription of *pmrD*, which in turn upregulates the PmrAB two-component system. (Adapted from Groisman, 2001.)

mutant, *pho*-24, demonstrated differential expression of approximately 40 proteins by two-dimensional gel electrophoresis ((Miller and Mekalanos, 1990). Like the *pags*, proteins encoded by PhoP-repressed genes (*prg*) are equally important for *Salmonella* pathogenesis. For example, PrgH contributes to *Salmonella* virulence by both the oral and intraperitoneal routes, and *prgH* mutants are defective in bacterium-mediated uptake by epithelial cells (Behlau and Miller, 1993). This suggests that PhoP-repressed loci may be important early in *Salmonella* infection, while PhoP-activated loci are important during later stages of infection, such as intramacrophage survival. This hypothesis was further supported when PrgH was identified as the base of the type III secretion system located on the *Salmonella* pathogenicity island, SPI-1 (Kubori et al., 1998). Indeed, it was the characterization of the PhoP-repressed genes that led to the identification of the type III secretion apparatus in *Salmonella* (Klein et al., 2000; Kubori et al., 1998, 2000; Pegues et al., 1995; Sukhan et al., 2001, 2003).

In addition to cellular invasion and intracellular survival, PhoP appears to regulate other adaptations for *Salmonella* infection and persistence in its host. For example, activation of PhoP-PhoQ coincides with the downregulation of flagellar genes, and a mutant that constitutively expresses PhoP is less motile (Adams et al., 2001; Bader et al., 2003). The full repertoire of PhoP-PhoQ-regulated virulence genes and their function in *Salmonella* remains to be elucidated.

4.1.6. *Pseudomonas aeruginosa* PhoP-PhoQ

Initial attempts to identify PhoP-PhoQ homologs in *P. aeruginosa* by Southern hybridization failed (Groisman et al., 1989). However, Macfarlane et al. (1999) identified a PhoP-PhoQ homolog in *P. aeruginosa* that is 53% and 33% identical to PhoP and PhoQ of *Salmonella* and *E. coli*, respectively. In *P. aeruginosa*, PhoP-PhoQ is located immediately downstream of the gene-encoding OprH—a major outer-membrane protein that is upregulated in response to low Mg^{2+}. RT-PCR and Northern blot analyses demonstrated that *oprH*, *phoP*, and *phoQ* composed a single transcript, and transcriptional fusions to the xylene reporter gene confirmed that the operon was transcriptionally regulated in response to extracellular Mg^{2+} via PhoP-PhoQ (Macfarlane et al., 1999). Such PhoPQ regulation of a transcriptionally linked gene is distinct from that observed in other bacterial species harboring PhoP-PhoQ.

The molecular characterization of PhoP-PhoQ in *P. aeruginosa* has only recently been addressed. Initial observations suggest both similarities and differences in the function of PhoPQ in *Pseudomonas* versus that in *E. coli* and *Salmonella*. As observed in *E. coli* and *Salmonella*, transcription of the *oprH-phoP-phoQ* operon in *P. aeruginosa* is regulated by extracellular Mg^{2+} levels (Macfarlane et al., 1999, 2000). In the absence of PhoQ, PhoP appears to activate OprH expression in both low and high magnesium,

while PhoQ restores the downregulation of PhoP in response to high concentrations of Mg^{2+}. Interestingly, PhoP is capable of activating significant levels of OprH production in the absence of PhoQ. This suggests that PhoP may be phosphorylated by an HK in addition to PhoQ. The possibility that PhoPQ in *P. aeruginosa* is highly integrated with other TCRs (such as GacS-GacA and PrmA-PmrB—see below) seems likely considering the presence of 63 sensor kinases and 64 response regulator genes in this organism (Rodrigue et al., 2000). In a separate study, the sensor GacS was shown to overlap with PhoQ in regulating several virulence determinants in *P. aeruginosa*, including swarming, lipase production, and virulence in mice (Brinkman et al., 2001).

Unlike PhoPQ in *Salmonella*, PhoPQ does not appear essential for antimicrobial peptide resistance in *P. aeruginosa*. Mutants of *phoP* and *phoQ* remain resistant to polymyxin under Mg^{2+}-deficient growth conditions as a result of PmrA-PmrB-mediated resistance (Macfarlane et al., 1999; Moskowitz et al., 2004). However, PhoPQ contributes to the antimicrobial resistance of *P. aeruginosa* indirectly via interaction with PmrA-PmrB and directly through regulation of an LPS modification operon (PA3552-PA3559) that is homologous to the *pmr* operon of *Salmonella* (Macfarlane et al., 2000; McPhee et al., 2003). The precise mechanisms of antimicrobial resistance and the respective roles of PhoPQ and PmrAB are currently under investigation.

The importance of PhoP-regulated LPS modifications in bacterial pathogenesis is probably most evident in chronic *P. aeruginosa* infections. *P. aeruginosa* clinical isolates from the lungs of cystic fibrosis (CF) patients contained modified lipid A structures characterized by the addition of aminoarabinose and palmitate to lipid A (Ernst et al., 1999). These modifications to LPS were associated with increased resistance to cationic antimicrobial peptides similar to that observed for *Salmonella* grown in low Mg^{2+} in vitro. In addition, the modified lipid A isolated from CF patients was a more potent inducer of the innate immune response in vitro. A *phoP* null mutant was unable to modify the lipid A moiety, thus suggesting a critical role for PhoP in the pathogenesis of *P. aeruginosa* in the CF lung (Ernst et al., 1999).

4.1.7. *Shigella flexneri* PhoP-PhoQ

The *phoP* gene of *Shigella* is 100% identical to *E. coli phoP. PhoP* mutants in *Shigella* have decreased resistance to polymorphonuclear leukocytes and antimicrobial peptides, but are not defective in epithelial cell invasion, intracellular growth, or acid tolerance. In the murine pulmonary model of infection, *phoP* mutants colonize the tissue with similar efficiency but are cleared from the lungs earlier than the wild-type strain. Furthermore, the inflammation induced by *phoP* mutants in a keratoconjunctivitis model is resolved more quickly than that induced by wild-type *Shigella* (Moss et al., 2000). The PhoPQ regulon in *Shigella* remains to be elucidated.

4.1.8. *Yersinia pestis* PhoP-PhoQ

PhoP orthologs have been cloned and sequenced in *Y. pestis*, *Y. pseudotuberculosis*, and *Y. enterocolitica*. PhoP in *Y. pestis* is 82% and 80% identical to PhoP in *E. coli* and *Salmonella*, respectively. Like *E. coli* PhoP, the conserved aspartate residue is found at position 51 in *Y. pestis*. Mutations in PhoP render *Y. pestis* more sensitive to high osmolarity, low pH, and oxidative killing. In addition, *Y. pestis* PhoP mutants have a reduced ability to survive in J774 macrophages and are less virulent in mice (Oyston et al., 2000). Two-dimensional gel electrophoresis indicates that PhoP regulates approximately 13 proteins differentially at both 28˚C and 37˚C; however, the PhoPQ regulon in *Y. pestis* (as well as the other *Yersinia* pathogens) remains to be characterized.

4.1.9. *Neisseria meningitidis* PhoP-PhoQ

A *phoP* homolog in *N. gonorrhoeae* was first identified by Southern hybridization (Groisman et al., 1989). PhoP-PhoQ was later shown to be present and functional in *N. meningitidis* as well (Johnson et al., 2001). PhoP and PhoQ of *N. meningitidis* are 34% and 25% identical to PhoP and PhoQ of *Salmonella*. Meningococcal *phoP* mutants contained differences in polypeptide profile, displayed poor growth at low concentrations of Mg^{2+}, and had increased sensitivity to defensins. In addition, the *phoP* mutant was deficient for growth in murine serum, suggesting a sensitivity to complement as well. Interestingly, *phoP* mutants were attenuated in their ability to translocate across a human epithelial cell monolayer (Johnson et al., 2001). When *phoP* was disrupted in a virulent serogroup C meningococcal strain, the mutant was completely avirulent in the mouse upon intraperitoneal challenge (Newcombe et al., 2004). The PhoP-regulated virulence genes in *Neisseria* remain to be discovered.

4.2. BvgAS, a Two-Component Regulatory System in Bordetella pertussis

4.2.1. The Bvg-regulon

The pathogenesis of infection by *B. pertussis*, the etiologic agent of whooping cough, involves the expression of several virulence factors including toxins such as pertussis toxin (Ptx), adenylate cyclase toxin (Cya), dermonecrotic toxins (Dnt), the major adhesin filamentous hemagglutinin (Fha), tracheal colonization factor (Tcf), and the outer-membrane protein pertactin (Prn) (Finn and Stevens, 1995; Leininger et al., 1991; Makhov et al., 1994; Weiss et al., 1984). Genes encoding these virulence factors in *B. pertussis* are all regulated coordinately at the transcriptional level by the master TCR BvgAS. In 1960, Lacey (1960) reported that expression of several virulence determinants of *B. pertussis* was altered by changing growth

conditions, a phenomenon now referred to as "phenotypic modulation." Other investigators observed that addition of $MgSO_4$ or nicotinic acid to the growth medium or lowering the growth temperature resulted in an inhibition of expression of virulence-associated genes (Melton and Weiss, 1989; Miller et al., 1992a). A second mechanism was termed "phase variation" and represented a spontaneous usually irreversible loss at high frequency of virulence gene expression that resulted in avirulent variants of *B. pertussis* (*vir*$^-$), although revertants to a virulent phase (*vir*$^+$) were also observed (Leslie and Gardner, 1931; Monack et al., 1989; Stibitz et al., 1989; Weiss et al., 1984). Both of these phenomena, "phenotypic modulation" and "phase variation," were originally recognized as regulated by the *bvg* (*b*ordetella *v*irulence *g*enes) locus. Early evidence demonstrated that expression of several virulence-associated genes was eliminated in avirulent phase variants containing a Tn5 insertion in the *bvg* locus, generated by transposon mutagenesis (Weiss et al., 1983, 1984). Other authors reported that *bvg* mutants were unable to demonstrate phenotypic modulation, and strains constitutively expressing virulence factors contained mutations in the *bvg* operon (Gross and Rappuoli, 1989; Miller et al., 1992a). Thus, under nonpermissive conditions, i.e., in response to a modulating signal or due to mutations that inactivate the *bvg* locus, the organism reacts with a switch from a virulent Bvg$^+$ phase to a nonvirulent Bvg$^-$ phase.

4.2.2. Signal Transduction Domains in BvgAS

The *bvg* operon encodes three proteins: BvgA, a DNA-binding response regulator; BvgS, a transmembrane sensor protein residing in the cytoplasmic membrane; and BvgR, a 32 kDa repressor protein (Merkel et al., 2003). BvgA is a 23 kDa traditional response regulator with an N-terminal receiver domain and a C-terminal helix-turn-helix DNA-binding domain (Arico et al., 1989). BvgS is a 135 kDa integral cytoplasmic membrane protein with kinase activity, initially reported as the product of two genes, *bvgB* and *bvgC*, but was later found to be encoded by a single gene, termed *bvgS* (Stibitz and Yang, 1991). Although, the BvgS protein shares sequence homology with proteins of a classical TCR, the cytoplasmic region differs in that it contains additional signaling modules apart from the transmitter domain (Figure 6). The *bvg* operon has in accordance been classified as a member of an "unorthodox" subfamily of TCRs referred to as a phosphorelay system (Perraud et al., 1999; Uhl and Miller, 1996b). The additional signaling domains, the receiver and the HPt, can both be combined in a single protein (polydomain) in the C-terminus of BvgS or they can be separated on different protein molecules as in the *B. subtilis* sporulation Kin/Spo phosphorelay system (Burbulys et al., 1991).

In BvgS, the transmembrane domain is followed by a linker region, recently identified by sequence homology as a putative PAS domain, which is followed by the transmitter domain containing a conserved histidine residue

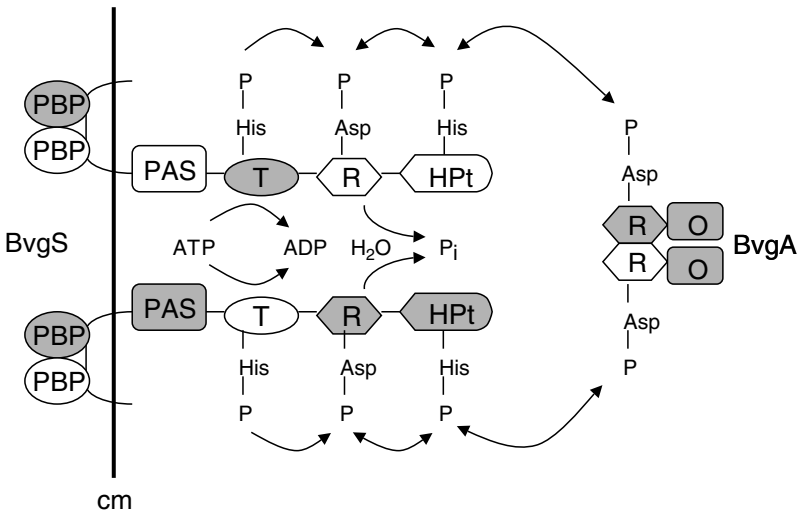

FIGURE 6. Domain structure of the BvgAS two-component phosphorelay system. Both the BvgS and the BvgA proteins are dimerized. Each BvgS monomer contains a polydomain structure composed of two binding protein domains located in the periplasm. The PAS domain is connected to a transmitter domain and a receiver domain, and the histidine-containing phosphotransfer domain. Upon activation of the autokinase, ATP is hydrolyzed at the ATP subdomain of the transmitter, which results in phosphorylation of the histidine residue. A γ-phosphate is then transferred via an aspartic acid residue of the receiver domain to a histidine residue of the C-terminal phosphotransfer domain. Activation of BvgA is achieved by transfer of the phosphoryl group to an aspartic acid residue located in the receiver domain. CM, cytoplasmic membrane; HPt, histidine-containing phosphotransfer domain; O, output domain; PAS, PAS domain; PBP, periplasmic binding protein; R, receiver; T, transmitter. (Modified from Cotter and Jones, 2003.)

(His729) that is the available target for autophosphorylation (Figure 6) (Boucher et al., 1994; Taylor and Zhulin, 1999; Uhl and Miller, 1994). This type of system is characterized by an intramolecular transfer of a γ-phosphoryl group of ATP in a multistep process via His-Asp-His-Asp residues upon activation of the sensor protein by the incoming environmental signal through the N-terminal region located in the periplasm (Figure 6). The phosphate is transferred to the receiver domain (Asp1023), to the C-terminal HPt domain (His1172), and finally to an aspartic acid residue (Asp54) in the receiver domain of BvgA (Uhl and Miller, 1996b), which increases its affinity for virulence-associated promoters (Steffen et al., 1996). Control of virulence gene expression may be explained by the fact that the HPt domain functionally can interrupt the phosphoryl group transfer by hydrolytically releasing inorganic phopsphate, which may affect the phosphorelay and thereby decrease intracellular amounts of BvgA-P (Uhl and Miller, 1996a).

Furthermore, genetic and biochemical evidence indicate that both BvgA and BvgS likely have dimerization properties, which may functionally facilitate phosphorylation between two protein homodimers (Beier et al., 1995; Scarlato et al., 1990). This domain structure may illustrate a mechanism whereby the receiver activity of BvgS could vary whether both, one, or none of the dimers is phosphorylated, suggesting a function more like a rheostat than a switch of the BvgAS system. In general, a phosphorelay system is considered a more complex signal transduction mechanism, and additional members of this subfamily that have hitherto been identified containing polydomain sensors and regulating virulence gene expression include the following: GacAS (*P. aeruginosa*), BarA/SirA (*S. enterica*), LetAS (*Legionella pneumophila*), and ArcAB (*E. coli*) (Altier et al., 2000; Georgellis et al., 1998; Hammer et al., 2002; Heeb and Haas, 2001).

4.2.3. Transcriptional Control of the *bvg* operon and Associated Virulence Genes

Under permissive conditions when the organism is in the virulent Bvg^+ phase, the *bvgAS* operon activates transcription of *vag* genes (*v*irulence-*a*ctivated *g*enes); under nonpermissive conditions the organism is in a nonvirulent Bvg^- phase and represses transcription of *vrg* genes (*v*irulence-*r*epressed *g*enes). Transcriptional repression is achieved by expression of a recently discovered negative regulator protein BvgR, which is under positive transcriptional control of the *bvgAS* operon (Merkel and Stibitz, 1995). Transcription of *bvg*-activated promoters is differentially regulated and has been classified as "early" or "late" based on the kinetics of activation following transcription of the *bvg*, *fha*, *ptx*, and *cya* promoters by an inducing signal. The early class is represented by *bvg* and *fha* and the late class is represented by *ptx* and *cya*. Also, the phosphorylated form of BvgA is known to have an increased affinity for *bvg*-dependent promoters such as *ptx* (Boucher and Stibitz, 1995; Zu et al., 1996), *cyaA* (Karimova et al., 1996), and *fha* (Roy and Falkow, 1991; Steffen et al., 1996; Zu et al., 1996), and is therefore required for expression of these promoters. Transcription of the *bvg* operon is known to be autoregulated and controlled by a 350 bp DNA fragment that contains five overlapping promoters comprising a 427 bp intergenic sequence between two divergently transcribed genes, *bvgA* and *fha* (Roy et al., 1990; Scarlato et al., 1990; Uhl and Miller, 1994).

The transcriptional start point for these promoters has been determined, whereby it was demonstrated that three promoters, P_1, P_2, and P_3, regulated the transcription of *bvgA* and *bvgS* (Roy et al., 1990; Scarlato et al., 1990). One promoter directs the synthesis of antisense RNA complimentary to RNAs synthesized by the P_1, P_2, and P_3 promoters. The fifth promoter, P_{FHA}, regulates the transcription of the divergently oriented gene, *fha*, which is positively regulated by the *bvg* operon (Stibitz et al., 1988). Under nonpermissive conditions only the P_2 promoter is transcribed generating a low basal level of

regulatory protein *bvgA*, which is not in an active form to initiate the transcription of virulence-associated genes. However, under physiological relevant conditions (i.e., body temperature vs. a colder millieu), *bvgS* is autophosphorylated and this induces an activation of BvgA through phosphorylation that presumably generates an active dimer of BvgA-P (Beier et al., 1995; Scarlato et al., 1990). Sufficient amounts of biologically active BvgA are now available to initiate transcription of all *bvg*-regulated virulence genes via the activation of the *bvg* promoters P_1, P_3, and P_4. Under these conditions the activation of the BvgA protein was increased approximately 50-fold, which is sufficient for complete activation (Scarlato et al., 1990). Later, a mechanistic characterization of Bvg-activated promoters was conducted by S1 nuclease protection assays, gel retardation, and footprinting experiments.

These analyses have evidenced that BvgA-P interacts and binds to an upstream primary binding site in the promoter regions of *bvg*, *fha*, *ptx*, and *cya*, respectively (Boucher and Stibitz, 1995; Karimova et al., 1996; Steffen et al., 1996; Zu et al., 1996). The derived consensus sequence consists of heptanucleotide inverted repeats of TTTC(C/T)TA with different spacing between the two halves of the repeat, which is presumed to be the initial primary binding site for phosphorylated BvgA (Roy and Falkow, 1991). Transcription of *fha* is activated by binding of a BvgA dimer to the primary high-affinity binding site that is centered at position –88.5 relative to the transcriptional start site in the *fha* promoter region (Roy and Falkow, 1991). This is followed by cooperative binding of an additional BvgA dimer to a secondary region downstream of the primary binding site within the *fha* promoter, where BvgA binds with lower affinity for complete activation and functional recruitment of RNA polymerase (Boucher et al., 1997, 2001).

The nucleotide organization and mechanism of activation of the *ptx* promoter is similar to that of the *fha* promoter, although the initial BvgA binding site is situated further upstream centered at position –136.5 relative to the transcriptional start site and matches the consensus sequence at only 10 of 14 bp. Activation of the *ptx* promoter requires binding of two BvgA dimers and subsequent cooperative binding of multiple BvgA dimers downstream analogous to the *fha* promoter region (Boucher and Stibitz, 1995; Marques and Carbonetti, 1997). Since the phosphorylated form of BvgA demonstrates a higher affinity for individual promoters than its unphosphorylated form, it has been demonstrated that an early promoter such as the *fha* promoter needs a lower concentration of BvgA-P for its activation compared to the late *ptx* and *cya* promoters. It has also been reported that an accumulation of cytoplasmic BvgA-P correlated with transcription of the two late promoters *ptx* and *cya* (Scarlato et al., 1991; Steffen et al., 1996; Zu et al., 1996).

The outer-membrane protein pertactin is encoded by *prn* and is involved in mammalian cell adherence and invasion. Recently, it was demonstrated that transcription of *prn* is dependent on the phosphorylated form of BvgA and that BvgA binds to an upstream sequence, suggested as the primary BvgA binding site with homology to similar sequences found in promoters of *bvg*,

fha, *ptx*, and *cya*. It was also concluded from activation kinetics studies that transcription of the *prn* promoter may be classified as a third intermediate group of Bvg-activated promoters since transcripts of *prn* was detected at a later time point than *fha* but earlier than *ptx* (Kinnear et al., 1999). Thus, differences in the kinetics of activation and the relative strength of the above discussed BvgA-activated promoters are dependent not only on available concentrations of phosphorylated BvgA and the phosphorylation state of BvgA-P but also on the affinity of BvgA-P for the primary binding site as well as the total number of secondary low-affinity binding sites within these promoters (Gross and Rappuoli, 1989; Scarlato et al., 1991; Steffen et al., 1996; Zu et al., 1996).

The function and regulation of Bvg-repressed genes in *B. pertussis* is basically unknown. A transcriptional repressor protein encoded by *bvgR* has been identified that is required for the regulation of *vrg* genes (Merkel and Stibitz, 1995). The *bvgR* locus is located downstream of *bvgAS*, is divergently transcribed relative to *bvgAS*, and transcription of *bvgR* has been shown to be dependent on BvgA (Merkel et al., 1998). Interesting differences in the regulation and expression pattern have recently been discovered by an extensive characterization of *bvgR* (Merkel et al., 2003). Phosphorylation of BvgA was required for transcriptional activation of the *bvgR* promoter. DNase I footprinting analysis evidenced a primary BvgA binding site that was located adjacent to the RNA polymerase binding site and a secondary binding site located upstream of the primary binding site. The BvgA binding site consisted of an unsymmetrical half of the known inverted repeat consensus sequence. Finally, it was concluded that BvgA-P interacted first with the downstream binding site and thereafter with the more upstream site, and that occupation of both sites was needed for transcription. This is in contrast to all previously described Bvg-activated promoters in *B. pertussis*.

4.2.4. The Third Intermediate Phase, Bvg-intermediate Phase (Bvg^i)

The mechanism of the BvgAS signal-transduction system in *B. pertussis* and of other TCRs in general has traditionally been considered a sensory-regulatory modulator of virulence that responds to environmental stimuli by turning transcription of certain necessary genes on and off, thus acting as a switch. Recently, however, a *bvgS* mutant was isolated that manifested a reduced ability to colonize the rat respiratory tract but had gained ability to survive in a nutrient-limited growth media (Cotter and Miller, 1997). This discovery indicated that a third phenotypic phase was evident in *B. bronchiseptica*, a Bvg-intermediate phase (Bvg^i-phase). This Bvg^i phenotype carried a threonine to methionine substitution in the BvgS transmitter domain that resulted in an in-between concentration of BvgA-P. Accordingly, this led to the conclusion that the bvgAS operon had the capacity of responding to external stimuli by a range of phenotypic states. Under these conditions neither the Bvg-repressed genes nor the late Bvg-activated genes were trans-

cribed; only the early *bvgAS*, *fhaB*, and *ptx* genes were transcribed. An early indication was made by Scarlato et al. (1991) of a third phenotypic phase in *B. pertussis*, where they demonstrated that a modulating signal shifted the phenotype of the organism from a bvg^- phase to a bvg^+ phase while transiently expressing *fha*. Ten years later the first bvg^i phase protein BipA was identified and characterized along with its structural gene *bipA* (Stockbauer et al., 2001). An interesting observation revealed maximal expression of *bipA* during bvg^i phase conditions, and that the N-terminal of the protein composed of 1570 amino acids was homologous to surface exposed proteins intimin (Int) of *E. coli* and invasin (Inv) of *Yersinia* spp. However, the relevance of BipA as having a role in virulence in vivo is questionable based on observations that *bipA* was not required for either adherence to rat lung epithelial cells in vitro or colonization of rabbit respiratory tract in vivo. Nevertheless, conclusive evidence would suggest that Bvg^i phase-dependent gene expression represents a response of the BvgAS system to subtle changes in environmental conditions, which means that the BvgAS system has the capacity of working more like a rheostat than a true switch.

Microarray analysis is a powerful tool for assessing the global transcriptional profile of a gene pool in a specific experimental situation. This technique was recently utilized by Hot et al. (2003) to verify the effect of modulating signals on BvgAS-dependent regulation of all *vag* and *vrg* genes in *B. pertussis*. Interestingly, all previously known *vags* and *vrgs*, with the exception of one *vrg*, were confirmed as members as previously assessed. Additionally, a number of new genes were identified belonging to these classes, including genes encoding autotransporters, TCRs, and extracellular sigma factors among others. Expression of the BipA protein was reported as the only example that displayed a large level of variation of its expression pattern in response to different growth conditions. Surprisingly, *bipA* was identified as a *vag* gene by microarray analysis and RT-PCR but as a *vrg* gene by *lacZ* fusion analysis, indicating that as a Bip^i phase gene, regulation of *bipA* gene expression is complex and sensitive to subtle environmental variations.

5. Conclusions

Two-component regulators (TCRs) are critical for the control of virulence determinants in both Gram-negative and Gram-positive organisms. It is becoming apparent from the plethora of bacterial genomic sequences that are currently available that TCRs are required for versatility in gene expression. This is evidenced by the fact that bacteria that undergo more complex lifestyles (e.g., soil and water microorganisms and bacteria that sporulate) contain more TCRs. It is also becoming clear that the modules for TCRs are more conserved at their structural level but evolution has modified them over time to accommodate a vast range of responses. Moreover, there is

conservation across several genera of specific TCRs. For instance, the contribution of PhoP-PhoQ to virulence has been established in several Gram-negative pathogens and appears species-specific. Therefore, the identification and characterization of regulated loci in each pathogen by specific TCRs may provide novel targets for antimicrobial therapy as well as further our understanding of pathogenesis.

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Chapter 14

Oxidative Stress Systems in Bacteria: Four Model Organisms

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Historical Landmarks

1876 Fenton reports a reaction between iron salts, hydrogen peroxide, and tartaric acid (Fenton, 1876). Ultimately, the reaction, which today bears his name, was demonstrated to be mediated by hydroxyl radical.

1900 Loew describes the existence of an enzyme, for which he proposes the name catalase, that catalyzes the degradation of hydrogen peroxide (Loew, 1901). He speculates that it exists to protect cells against H_2O_2, which might be made as a by-product of cellular metabolism.

1934 Neuman, under the direction of Linus Pauling, demonstrates the stable existence of superoxide, and he proposes its name (Neuman, 1934).

1934 Haber and Weiss discover the iron/copper-dependent degradation of H_2O_2 (Haber and Weiss, 1934). Superoxide is an intermediate.

1956 Fridovich finds that a product derived from liver extracts, hypoxanthine, is able to drive the sulfite-dependent xanthine oxidase reaction (Fridovich and Handler, 1956)

1949 Xanthine oxidase is shown to catalyze the oxygen-dependent-reduction of cytochrome c (Horecker and Heppel, 1949).

1969 Discovery of SOD. The simple xanthine oxidase reaction formed the basis for what is the most commonly used assay for SOD, the SOD-inhibitable xanthine oxidase-hypoxanthine catalyzed reduction of cytochrome c (McCord and Fridovich, 1969). McCord and Fridovich in 1969 described the function of what was formerly known as erythrocuprein.

1970 Discovery that *Escherichia coli* harbors a Mn-SOD (Keele et al., 1970).

1971 An activity stain for SOD (Beauchamp and Fridovich, 1971).

1971 McCord et al. report a correlation between bacterial aerotolerance and titers of SOD and catalase (McCord et al., 1971).

1973 Discovery of an iron-containing SOD (Yost and Fridovich, 1973).

1974 Discovery of a bacterial CuZnSOD (Puget and Michelson, 1974).

1976 Boehme et al. demonstrate that hyperoxia toxifies *E. coli* by blocking specific steps in several amino acid biosynthetic pathways (Boehme et al., 1976).

1976 Discovery that phagocytes generate O_2^- and H_2O_2 (Babior and Kipnes, 1976).

1977 Paraquat, or methyl viologen, was used as an herbicide in 1969 when the then president, Richard M. Nixon, initiated the program "Operation Intercept." This was intended to thwart the sale and use of marijuana in the U.S. However, marijuana farmers in Mexico

still attempted to sell the paraquat-laced plants. Those unfortunate enough to have smoked or injested the contraband suffered from severe nosebleeds, headaches, and breathing disorders. Death from paraquat-laced marijuana was rare. However, paraquat was discovered to be extremely useful in the laboratory because of its capacity to redox-cycle. Hassan and Fridovich used paraquat to examine induction of Mn-SOD in *E. coli* (Hassan and Fridovich, 1977a).

1979 Claiborne and Fridovich discovered that *E. coli* possesses two catalases, one of which, HP-I, also has peroxidase activity (Claiborne and Fridovich, 1979; Claiborne et al., 1979).

1981 *Lactobacillus plantarum*, an aerobe, was found to lack SOD but accumulate high levels of manganese, which serve to protect these organisms from oxidative stress (Archibald and Fridovich, 1981).

1983 First cloning of a catalase gene from *E. coli* (Loewen et al., 1983).

1983 First cloning of a SOD gene, *sodA*, from *E. coli* (Touati, 1983).

1984 Cloning of the *sodB* gene of *E. coli* (Sakamoto and Touati, 1984).

1984 First isolation of catalase-deficient mutants of *E. coli* (Loewen, 1984).

1985 Cloning of *E. coli katG* gene, encoding the bi-functional catalase-peroxidase, HP-I (Loewen et al., 1985).

1985 Discovery of *E. coli* OxyR (Christman et al., 1989).

1986 Isolation of SOD-deficient mutants of *E. coli*, and documentation of their aerobic growth defects (Carlioz and Touati, 1986).

1987 Discovery that the catalytic iron–sulfur cluster of dihydroxyacid dehydratase is acutely sensitive to superoxide (Kogoma et al., 1988). Recognition that this is an attribute of many similar enzymes followed (Flint et al., 1993; Fridovich, 2003; Fridovich and Handler, 1956; Kogoma et al., 1988; Liochev and Fridovich, 1992).

1989 Discovery of alkylhydroperoxide reductase, a bacterial peroxidase (Jacobson et al., 1989).

1990 Discovery of *E. coli* SoxR by the B. Demple and B. Weiss labs (Greenberg et al., 1990; Tsaneva and Weiss, 1990).

1990 Role for peroxynitrite in oxidative damage is proposed (Beckman et al., 1990).

1992 Discovery of *E. coli* Dps (Almiron et al., 1992).

1998 Short chain sugars as generators of O_2^-. Glyceraldehyde, dihydroxyacetone, and erythrose inhibited growth of an *E. coli sodA sodB* mutant (Benov and Fridovich, 1998).

1999 Discovery of superoxide reductases among anaerobic bacteria (Almiron et al., 1992; Lombard et al., 2000).

1. Introduction

Over the last 30 years, oxidative stress has been the subject of energetic inquiry in mammalian systems, because the action of reactive oxygen species (ROS) has been linked to multiple health disorders. These include ischemia reperfusion injury, arthritis, coronary artery disease, amyotrophic lateral sclerosis (Lou Gehrig's disease), autoimmune and neurodegenerative diseases, stroke, and cancer. Further, the consequences of oxidative damage loom large and all too obvious in the form of wrinkles, thereby stimulating the creation of a vanity industry that has popularized the notion of oxidative stress on a more trivial level. However, despite enormous efforts, investigators working in mammalian systems have struggled to develop a clear molecular description of the nature of oxidative stress and therapies against it. The exceptions have been the recommended inclusion of both water-soluble (ascorbate, carotenoids) and fat-soluble (α-tocopherol) vitamins in our diets that have been shown to help slow the aging process. Instead, by virtue of their ease of metabolic and genetic manipulation, many of the molecular breakthroughs regarding oxidative stress mechanisms have occurred through studies in bacteria, due to both their methodological tractability and their responsiveness to environmental manipulation. *Escherichia coli* has served as the paradigmatic organism for investigations into the genesis and reactivity of ROS, and genetic analyses have revealed many of the strategies by which cells defend themselves. Studies in the opportunistic pathogen *Pseudomonas aeruginosa* and two important intracellular pathogens, *Salmonella typhimurium* and *Mycobacterium tuberculosis*, have offered instructive counterpoints, revealing commonalities and contrasts in defensive systems that reflect distinct lifestyles and niches of these four important organisms. For these reasons, this chapter will discuss bacterial oxidative stress mechanisms using these four microbes as model organisms.

2. Oxygen Toxicity and Reactive Oxygen Species

When oxygenic photosynthesis filled the primordial environment with molecular oxygen, it presented an opportunity for organisms to evolve highly efficient pathways for energy generation. However, the new aerobic lifestyle was not without its perils, and organisms routinely faced death because they were not prepared for the "new" gas that permeated their microcosm. It is likely that each organism has evolved only enough oxygen tolerance to thrive in its particular niche: contemporary organisms have difficulty tolerating oxygen concentrations much in excess of those that pervade their native habitats. However, while the fact of oxygen toxicity is plain to see, its molecular basis is not immediately obvious. Dioxygen itself (O_2) does not react with most biomolecules. In 1969, McCord and Fridovich made a monumental discovery

that opened the door to an understanding of this phenomenon: they isolated from bovine erythrocytes an enzyme, formerly known as erythrocuprein, which degraded O_2- to H_2O_2 and oxygen (McCord and Fridovich, 1969). Subsequent studies revealed that the enzyme, which they dubbed superoxide dismutase (SOD), is, in the vast majority of cases, ubiquitous among organisms that grow in oxygen-containing environments (McCord et al., 1971). They inferred both that O_2- is formed inside cells and that, if not scavenged, it could damage cellular components. The existence of enzymes that scavenge H_2O_2 had long been recognized, which led to an analogous inference about endogenous H_2O_2 and its downstream reduction product, the extremely reactive hydroxyl radical, $HO^{\cdot}$. The reactivity of these partially reduced oxygen species suggested that they might lie at the root of oxygen toxicity.

However, early efforts to confirm the ability of O_2- to damage biological molecules were unsuccessful (Bielski and Richter, 1977; Sawyer and Valentine, 1981) and elicited the suggestion that the dismutase activity of SOD might be adventitious (Fee, 1982). Yet further investigation established that SOD is a kinetically superior enzyme, reacting at virtually diffusion-limited rates with its substrate, O_2- (Getzoff et al., 1983). This seemed improbable for an accidental activity. The expression pattern also matched its proposed function. That is, SOD activity was induced when *E. coli* was exposed to hyperoxia or redox-cycling antibiotics, compounds that enter the cell and catalyze electron transfer from redox enzymes to molecular oxygen, causing profuse O_2- formation (Gelvan, 1997; Hassan and Fridovich, 1977b, 1977c; Hassett et al., 1987, 1992, 2000; Stadtman and Wittenberger, 1985). Further, this induction seemed to diminish the toxicity of those agents. While this evidence was circumstantial, it presented a compelling argument that the physiological role of SOD was indeed to scavenge O_2- (Fridovich, 2003). The issue was settled by the construction of SOD-deficient mutants. *E. coli* has three SOD isozymes, iron- and manganese-containing enzymes in the cytosol (Fe-SOD and Mn-SOD; Keele et al., 1970; Yost and Fridovich, 1973), and a copper/zinc enzyme (Cu,Zn-SOD) in the periplasmic space (Benov and Fridovich, 1994). Fe-SOD and Mn-SOD are induced and repressed, respectively, by iron, a fitting arrangement that ensures that one isozyme is always available. Carlioz and Touati (1986) demonstrated that mutants lacking both cytosolic enzymes grow well anaerobically but exhibit profound defects when aerated. The defects were fully complemented by expression of the mammalian Cu,Zn-SOD gene, which encodes an enzyme that is structurally distinct from the *E. coli* Fe-SOD and Mn-SOD but shares their ability to scavenge O_2- (Natvig et al., 1987). Thus, the toxicity of O_2- and the proposed role of SOD in protection against it were confirmed.

A similar argument can be advanced with respect to H_2O_2. *E. coli* expresses two catalases, hydroperoxidases I and II (HPI and HPII) (Claiborne and Fridovich, 1979; Claiborne et al., 1979), and one NADH peroxidase, originally called alkylhydroperoxide reductase (Ahp) (Jacobson et al., 1989). HPI and Ahp are expressed at moderate levels in log-phase cells and are further

induced in response to H_2O_2 exposure, whereas HPII is induced as cells enter stationary phase. Ahp is the primary scavenger when H_2O_2 concentrations are low (Seaver and Imlay, 2001); however, because its catalytic cycle requires the cyclical association and dissociation of AhpC and AhpF proteins, its activity is saturated when intracellular levels of H_2O_2 rise above 10 μM. Further, Ahp is likely to be ineffective when cells are starved and, as such, cannot readily supply NADH as a cofactor. These deficiencies are evidently compensated for by the catalases, which efficiently degrade even millimolar H_2O_2 levels and do not require a reductant. Mutants that lack all three enzymes cannot scavenge H_2O_2 and grow poorly in aerobic media, indicating that *E. coli* generates enough endogenous H_2O_2 to poison itself (Seaver and Imlay, 2001).

3. How Do O_2- and H_2O_2 Arise Inside *Escherichia coli*?

The growth phenotypes of SOD-deficient and peroxidase-deficient *E. coli* indicate that potentially toxic amounts of O_2- and H_2O_2 are formed endogenously during aerobic growth. The severity of these growth defects increases with oxygen concentration (Boehme et al., 1976). Because traditional oxidase reactions are saturated by micromolar levels of oxygen, the apparent unsaturability of oxidant production indicates that most intracellular O_2- and H_2O_2 are formed as inadvertent products of chemical oxidation reactions. This observation also suggests that microorganisms can lessen their oxidant burden by dwelling in microaerobic environments (e.g., biofilms), which provide oxygen concentrations that support their respiratory chain but minimize the generation of these toxic species.

Efforts to identify the mechanisms of O_2- and H_2O_2 formation have focused primarily upon the aerobic respiratory chain. Because molecular oxygen has two spin-aligned unpaired electrons (Figure 1), it can only oxidize biomolecules in single-electron steps (Imlay, 2003). Molecular oxygen has a low univalent reduction potential (–0.16 V, Figure 2) so that it is a weak acceptor of a single electron; furthermore, most biomolecules, including reduced dinucleotides, are poor univalent electron donors. Thus, because oxygen is constrained to accept electrons at one time, but is poor at doing so, it can react with very few biomolecules at significant rates. Therefore, researchers focused upon the respiratory chain (Gonzalez-Flecha and Demple, 1995), which employs a series of facile univalent electron carriers, as a plausible source of both O_2- and H_2O_2. Indeed, both species were detected as by-products during electron flow through inverted membrane vesicles in vitro (Imlay and Fridovich, 1991; Messner and Imlay, 1999). Biochemical analyses ultimately revealed that molecular oxygen can "steal" electrons from the flavins of reduced dehydrogenases (Figure 3), at rates that depend upon the degree to which the flavin is exposed on the surface of the enzyme (Messner and Imlay, 2002). NADH dehydrogenase II, the primary entry point for electrons into the chain, is a particularly autoxidizable respiratory enzyme in *E. coli*. Approximately 0.1% of the electrons that flow

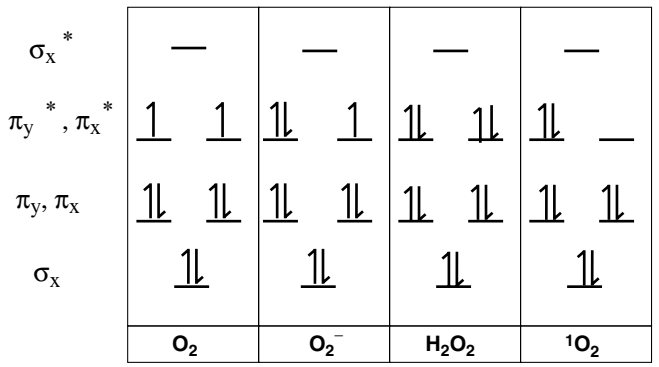

FIGURE 1. Schematic diagram of the molecular orbitals of O_2, O_2^-, H_2O_2, and $^1O_2$.

through the chain are intercepted by oxygen in vitro, forming a mixture of O_2- and H_2O_2 (Imlay and Fridovich, 1991). Although the fraction is small, extrapolation to the intact cell would predict that about 5 μM O_2- and H_2O_2 are generated per second in the cell (Imlay and Fridovich, 1991). However, actual measurements of H_2O_2 effluxing from cells that lack scavenging enzymes revealed that close to 15 μM H_2O_2 is generated per second (Seaver and Imlay, 2001), which suggested that another, yet-unidentified source predominates. Overproduction of NADH dehydrogenase II caused an increase in H_2O_2 efflux from such cells, confirming that it autoxidizes in vivo. However, mutants that lack NADH dehydrogenase II exuded near-wild-type levels of H_2O_2, indicating

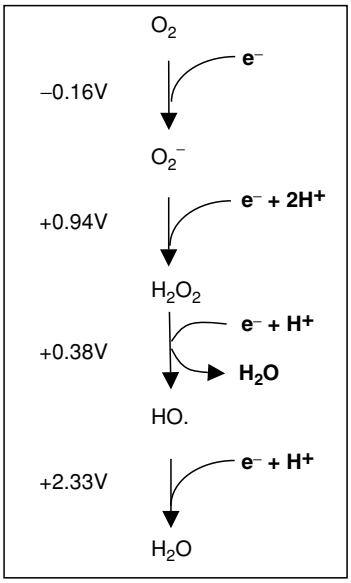

FIGURE 2. Standard reduction potentials for oxygen reduction species, assuming that 1 M dioxygen is used as the standard state for the first reduction.

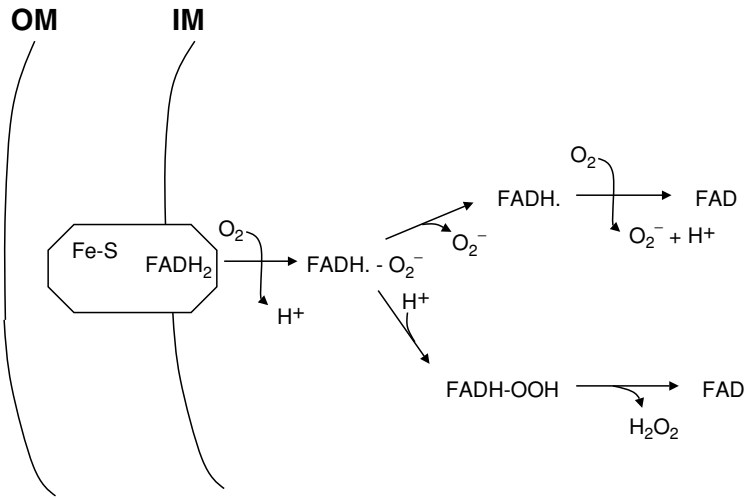

FIGURE 3. Autoxidation of flavoproteins can yield either superoxide (upper pathway) or hydrogen peroxide (lower pathway) as the immediate products. The overall rate of autoxidation depends upon the electron density on the flavin, the degree of flavin exposure to solutes, and the univalent reduction potential of the flavin.

that the primary source of H_2O_2 still remains to be discovered (Seaver and Imlay, 2004).

In any case, by considering the H_2O_2 production rate and the cellular content of scavenging enzymes, one can calculate that the steady-state level of H_2O_2 inside the wild-type cells must be about 20 nM. The level of O_2- is about 0.1 nM. These are the concentrations, then, which must be considered when evaluating the likelihood that intracellular oxidants damage biomolecules.

4. How Does O_2- Injure Cells?

Although O_2- was once thought to be physiologically benign, the poor growth and high mutation rate of SOD mutants (Carlioz and Touati, 1986) demonstrated that it is not. SOD-deficient mutants cannot grow without supplements of branched-chain, aromatic, and sulfur-containing amino acids, and they cannot catabolize TCA-cycle metabolites that are good growth substrates for wild-type bacteria. Further analyses revealed that O_2- inactivates dehydratases that employ solvent-accessible iron–sulfur clusters as Lewis acids. The branched-chain phenotype of the SOD mutants evidently results from the inactivation of dihydroxyacid dehydratase (Kuo et al., 1987), an enzyme midway in this pathway, while the TCA-cycle defect reflects the inactivation of aconitase (Gardner and Fridovich, 1991) and fumarase (Liochev and Fridovich, 1993). Cluster-containing dehydratases are found in other

organisms and presumably contribute to their sensitivity to oxidative stress. The rate constant for their oxidation by superoxide is remarkably high—10^6–10^7/M/s (Flint et al., 1993)—and should result in periodic inactivation of these enzymes even at the extremely low levels of O_2- that are found in SOD-proficient cells (Gort and Imlay, 1998).

The aromatic biosynthetic defect of SOD mutants may be caused by the ability of O_2- to oxidize an intermediate of the transketolase reaction, thereby aborting its catalytic cycle and blocking the synthesis of erythrose-4-phosphate, a precursor of aromatic amino acids (Benov and Fridovich, 1999) (Figure 4). The sulfur defect has been observed in SOD-deficient yeast as well (Chang and Kosman, 1990), but its molecular basis remains unclear (Benov and Fridovich, 1997).

5. How Does H_2O_2 Injure Cells?

While micromolar doses of H_2O_2 can inhibit growth of *E. coli*, the basis of this phenotype has been elusive, in large part because, until recently, the scavenging capacity of the cell has made it difficult to expose cultures to low-level H_2O_2 for an extended period of time. H_2O_2 has some ability to oxidize protein thiolates, but the rate constant is so low for typical cysteinyl residues (Winterbourn and Metodiewa, 1999) that for most proteins this reaction is

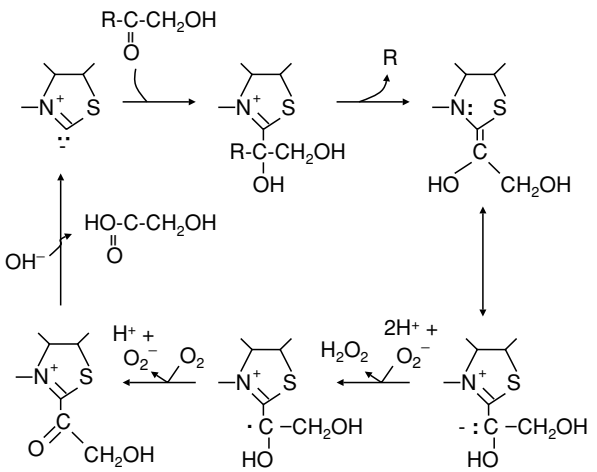

FIGURE 4. Scheme for the oxidation by superoxide of the dihydroxyethyl intermediate of the transketolase reaction cycle. Two valence structures for the dihydroxyethyl adduct are indicated by double arrowheads; only one valence structure of the radical intermediate is depicted. By causing glycolic acid to be released, the net effect of the oxidation would be to inhibit transketolation.

unlikely to be significant at physiological H_2O_2 levels. Further, while H_2O_2 can accelerate lipid peroxidation in mammalian cells, this process requires polyunsaturated fatty acids (Bielski et al., 1983), which are absent from most bacteria. Therefore, the growth-inhibitory action of H_2O_2 remains unsolved.

Higher concentrations of H_2O_2 cause cell death, due to the production of DNA lesions (Imlay et al., 1988). The immediate oxidant is not H_2O_2, which cannot react directly with DNA, but the highly reactive hydroxyl radical ($HO^{\cdot}$), which is the product of the Fenton reaction:

$$H_2O_2 + Fe^{2+} \rightarrow HO. + OH^- + Fe^{3+}$$

Cell-permeable iron chelators block oxidative DNA damage, confirming the role of iron in this process (de Mello Filho and Meneghini, 1985; Imlay et al., 1988).

The $HO^{\cdot}$ is an extremely powerful oxidant that reacts at diffusion-limited rates with the first organic molecule that it encounters. Thus, the radicals that damage DNA must be formed in its immediate vicinity. Iron binds DNA with high avidity (Rai et al., 2001), making this possible. Actively replicating *E. coli* harbor about 20 μM "free" iron; that is, iron that has not been incorporated into proteins and, as such, can participate in Fenton chemistry. Interestingly, O_2^- stressed cells contain up to an order of magnitude more free iron, apparently as a consequence of the destruction of iron–sulfur clusters and the consequent release of iron into the cytoplasm (Keyer and Imlay, 1996; Liochev and Fridovich, 1994). This liberated iron presumably is the basis of the high mutation rate of SOD-deficient mutants (Farr et al., 1986).

6. Inducible Cell Defenses Against O_2- And H_2O_2

The early observation that SOD levels increase in response to O_2- stress indicated that *E. coli* has a mechanism of sensing and responding to O_2-. Elegant independent studies by the Demple and Weiss laboratories revealed that *sodA* transcription is positively controlled by the SoxRS two-component system (Greenberg et al., 1990; Tsaneva and Weiss, 1990). O_2- oxidizes the iron-sulfur cluster of the SoxR protein, causing a change in the conformation of the protein and allowing it to productively bind the promoter of *soxS* (Ding and Demple, 1997; Gaudu et al., 1997). The SoxS protein amplifies this response by positively regulating a regulon that includes *sodA*, encoding the Mn-SOD. Other proteins that are induced as part of this response include fumarase C and aconitase A (Gruer and Guest, 1994; Liochev and Fridovich, 1992), stable dehydratases that replace their O_2^--sensitive isozyme counterparts.

O_2- levels within fully aerobic bacteria are normally insufficient to activate the SoxRS response, raising the question of what circumstance requires its action. In fact, redox-cycling drugs, such as those that are excreted by plants and competing microbes (e.g., *P. aeruginosa*, members of the Actinomycetales family, etc.) can elevate intracellular O_2- concentrations that are sufficient to

activate SoxR. These drugs include paraquat, plumbagin, menadione, streptonigrin, and many others (Chan and Weiss, 1987; Greenberg et al., 1990; Hassan and Fridovich, 1979; Kogoma et al., 1988). The AcrAB proteins, which comprise a drug export system, are components of the SoxRS response (White et al., 1997), which reinforces the notion that the system exists to stave off the oxidative stress that arises from such agents.

When *E. coli* is exposed to low doses of exogenous H_2O_2, it responds by inducing the synthesis of both catalase and peroxidase activities (Morgan et al., 1986). This adaptation is governed by the OxyR protein, a positive-acting transcription factor that is "primed" when H_2O_2 oxidizes an exceptionally reactive sulfhydryl group (Christman et al., 1985). In addition to scavenging enzymes, a variety of other proteins are upregulated as part of this response (Zheng et al., 2001a, b). Dps, an iron-binding protein, is among the most strongly induced. Its function is to sequester intracellular iron that might otherwise participate in the Fenton reaction (Ilari et al., 2002). While other regulated proteins have been identified, including glutathione reductase and iron–sulfur cluster assembly proteins, their particular contribution to H_2O_2 resistance has not yet been demonstrated.

7. Oxidative Stress In The *Escherichia coli* Periplasm

A periplasmic SOD isozyme was discovered in *Photobacterium leiognathi* in 1974 (Puget and Michelson, 1974). The enzyme contains copper and zinc and thus resembles the eukaryotic SODs more closely than the cytosolic iron- and manganese-containing bacterial enzymes that were known at the time. However, similar CuZnSODs were found in other bacteria in subsequent years, including a *sodC*-encoded enzyme in *E. coli* in 1994 (Benov and Fridovich, 1994). The *E. coli* enzyme eluded earlier discovery because it is expressed only in stationary phase (Imlay and Imlay, 1996). Although *sodC* mutants are slightly sensitive to H_2O_2, the phenotype has not yet pointed to the biomolecules that periplasmic SOD putatively defends. *S. typhimurium* has a second periplasmic Cu,ZnSOD that is important for its virulence (Vazquez-Torres et al., 2000), perhaps suggesting that these enzymes can defend bacteria against O_2- that is released as part of the oxidative burst of phagocytes (Babior et al., 1976). *S. typhimurium* oxidative stress systems will be discussed in greater detail in Section 16.

8. *Pseudomonas Aeruginosa*: An Obligate Respirer

E. coli resides in the microaerobic or anaerobic gut of humans and ruminants, and its infection cycle includes brief passage through sewage treatment systems, rivers, and contaminated streams. In contrast, the obligate respirer, *P. aeruginosa*, is a Gram-negative bacillus that is virtually ubiquitous in all natural settings. For example, it has been isolated from streams, lakes, oceans, ground wells, soil, and on plants and animals. It is also a pathogen of plants, animals,

various insects, the nematode *Caenorhabditis elegans*, and humans. It has received considerable notoriety because it is a pathogen of individuals whose immune systems have been compromised (e.g., cancer chemotherapy, AIDS, burn patients; Holder, 1993) or those afflicted with the autosomal inherited disease cystic fibrosis (CF) (see Hassett et al., 2002, 2003, 2004 for review). Complicating treatment strategies is the organisms' inherent ability to resist some biocides and several front-line antibiotics. We believe that the persistence of *P. aeruginosa* in problematic biofilms in environmental, industrial, and clinical settings (e.g., CF airways, colonization of catheters, heart valves, prostheses; Costerton et al., 1994, 1995, 1999; O'Toole, 2003) warrants an examination of physiological events that occur when the organism is under considerable stress. Maintaining an infection and/or a biofilm requires adaptive defense machinery that could be collectively grouped into what are commonly known as stress responses. In Sections 9–11, we examine several genes and gene products that are specifically involved in the oxidative stress response in *P. aeruginosa* and consider how this response might help maintain viability of the organism during infection and in biofilms. We also discuss both similarities and differences between *P. aeruginosa* and *E. coli* oxidative stress defense systems.

9. Oxidative Stress Systems In *Pseudomonas aeruginosa*

Similar to *E. coli*, *P. aeruginosa* generates its greatest metabolic energy through aerobic respiration. The organism is also capable of anaerobic growth via nitrate reduction and arginine substrate-level phosphorylation (Hassett et al., 2002). During aerobic respiration, *P. aeruginosa* must also defend itself from potentially toxic levels of reactive oxygen intermediates (ROIs). For example, defense against ROIs is provided by a large battery of scavenging enzymes: Fe-SOD and Mn-SOD (Hassett et al., 1993, 1995), three catalases (KatA, KatB, and KatC) (Brown et al., 1995; Ma et al., 1999), and at least three alkyl hydroperoxide reductases (AhpA, AhpB, AhpCF) and one organic hydroperoxide reductase (Ohr). What is currently known about these enzymes and their respective role(s) in oxidative stress defense will be discussed in detail below. However, we wish to emphasize here that the breadth of knowledge of mechanisms of oxidative stress and their defenses in *P. aeruginosa* is small compared to what is known of similar systems in *E. coli* that were discussed above and elsewhere (Imlay, 2003).

10. SOD

10.1. Fe-SOD

Fe-SOD is the primary "housekeeping" SOD in *P. aeruginosa* and is produced during all phases of aerobic or anaerobic growth (Hassett et al., 1992). In rich media, the typical specific activity for Fe-SOD alone is approximately

40 U/mg in cell extracts (Hassett et al., 1995). Because of this, a *sodB* mutant is sensitive to aerobic growth and O_2-generating agents (Hassett et al., 1999, 1995). The *sodB* gene is under partial regulatory control by the RhlR-RhlI quorum sensing system (Hassett et al., 1999) and responds positively to iron (Hassett et al., 1996). However, one of the most interesting phenotypes of a *sodB* mutant is an inability to produce the blue redox-active pigment pyocyanin during growth under phosphate-limiting conditions (Hassett et al., 1995). Part of the reason for this phenotype is that the increased level of O_2- in the *sodB* mutant poisons enzymes that are involved in the biosynthesis of pyocyanin. Pyocyanin itself generates O_2^-, and its role is to kill competitors. Thus, by employing superoxide-sensitive enzymes in the biosynthetic pathway, *P. aeruginosa* ensures that pyocyanin synthesis stops before it generates enough oxidative stress to harm the cell that makes it. Similarly, yet not surprisingly, pyocyanogenic bacteria have very high Fe-SOD activity (Hassett et al., 1992). This, too, makes perfect sense in that the organism ensures that the appropriate levels of oxidative stress protection in the form of Fe-SOD is abundant and functional so as to minimize the risk of self-damage.

10.2. Mn-SOD

The Mn-SOD of *P. aeruginosa* is encoded by the *sodA* gene, the last member of a four-gene operon starting with *fagA* (Fur-associated gene, an untranslated RNA), and including *fumC* (encoding stable fumarase) and *orfX* (unknown function) (Hassett et al., 1997). In contrast to *E. coli*, Mn-SOD activity is not detectable in aerobic mid-logarithmic phase organisms, but it can be detected in stationary phase aerobic cultures (Hassett et al., 1995). As in *E. coli*, the operon is also under negative control by the ferric uptake regulator (Fur) (Hassett et al., 1996). Fur is a dimeric repressor that contains iron and a corepressor (Ochsner et al., 2002). Thus, under iron-rich conditions, transcription of the operon is repressed; when iron is scarce, the operon is dramatically activated 119-fold as measured by Affymetrix microarray GeneChip, and 1120-fold as measured by *lacZ* fusion (Ochsner et al., 2002). The production of Mn-SOD likely serves to protect the organism against O_2- when limited intracellular iron concentrations reduce the amount of active Fe-SOD in the cell. This is similar to the iron-regulated activation of *sodA* in *E. coli* in the presence or absence of oxygen (Privalle and Fridovich, 1988).

Interestingly, the LasR-LasI quorum sensing circuit is also absolutely required for transcriptional activation of the *fagA-fumC-orfX-sodA* operon in that a *lasI* mutant was unable to produce Mn-SOD (Hassett et al., 1999). When the LasI gene product, *N*-3-oxo-dodecanoyl-homoserine lactone, was provided in the growth medium to this strain, Mn-SOD activity was restored (Hassett et al., 1999). Mn-SOD activity is also increased in biofilms, indicating that this mode of growth fosters an environment that elicits an iron-starvation response (Bollinger et al., 2001). However, transcriptional activation of Mn-SOD is independent of quorum sensing in biofilms, because

there appears to be a built-in "iron override" system in place to assure activation of the *fagA-fumC-orfX-sodA* operon in the absence of the *las* quorum sensing system.

11. Catalase

11.1. KatA

KatA in *P. aeruginosa* is a 170 kDa "housekeeping" catalase that is produced during all phases of aerobic or anaerobic growth, although its activity is maximal in the stationary growth phase and is ironically highest during anaerobic growth (Ma et al., 1999). KatA provides 95% of the cellular catalase activity; thus, a *katA* mutant is very sensitive to exogenous H_2O_2 (Hassett et al., 1999; Ma et al., 1999). KatA is the only catalase of its kind, a 170 kDa α_2,β hetero-trimer and a member of the Group III catalases (see Klotz et al., 1997; Loewen et al., 2000 for a review). The *katA* gene was found to contain two intragenic translational start codons, one at the position 1 methionine, the other residing 263 bp downstream. This was confirmed experimentally by both SDS-PAGE analysis of purified KatA and MALDI-TOF mass spectrometric analysis of trypsin cleavage peptides derived from KatA. Interestingly, an excellent overlapping Shine–Dalgarno sequence rests 5–10 bp upstream of the second translational start. Transcription of *katA* is driven, in part, by the general stress resistance σ factor, RpoS (Suh et al., 1999), which, in turn, is also controlled by the LasR-LasI and RhlR-RhlI quorum sensing systems (see below) (Hassett et al., 1999). Thus, it is not surprising that *rpoS* and quorum sensing–deficient mutants are more sensitive to H_2O_2 than are wild-type bacteria (Hassett et al., 1999; Suh et al., 1999).

Another factor essential for optimal KatA activity is the iron storage protein BfrA. The *bfrA* gene lies immediately downstream of *katA*, and both genes respond positively to iron and H_2O_2, yet are surprisingly not part of an operon (Ma et al., 1999). A mutant lacking BfrA produces only approximately 50% of the KatA activity of wild-type organisms, despite possessing wild-type *katA* transcription and translation product. The working hypothesis for this observation is that there is an as yet uncharacterized iron-trafficking mechanism whereby the iron that is loaded into the core of BfrA (up to 700 Fe atoms/BfrA; Moore et al., 1994, and 3–9 heme groups per BfrA 24-mer; Kadir and Moore, 1990) is reduced by the inherent ferroxidase activity of BfrA, and the iron that is then released is incorporated into protoporphyrin IX by ferrochelatase. The ferrochelatase reaction is the last step in the biosynthesis of heme, a required component of the vast majority of bacterial catalases. Mutation of amino acids E18K and Y25I in BfrA that are essential for ferroxidase activity reduced KatA activity to levels produced by a Δ*bfrA* mutant (Ma et al., 1999). Thus, what is commonly referred to as "free" iron is sequestered by BfrA, BfrB, and Dps, and DNA repair enzymes address any

lesions that nevertheless occur (Beyer et al., 1991; Imlay and Linn, 1987; Imlay et al., 1988; Ma et al., 1999). The mechanism by which the iron within BfrA is potentially "earmarked" for KatA is unknown, but the potential trafficking mechanisms are intriguing and certainly worthy of further study.

11.1.1. Role of KatA in Biofilm Protection Against H_2O_2

H_2O_2 has been used for over 50 years as an antimicrobial biocide, in part due to its ability to oxidize proteins and cause transition metal-catalyzed single-strand nicks in DNA. Yet in biofilms, we must envision a vastly different scenario. Because there are often steep oxygen gradients within mature biofilms (Xu et al., 1998), especially in the context of CF airway infection (Worlitzsch et al., 2002), the environment for organisms at or near the base of biofilms would, in theory, be oxygen-limited or even anaerobic. This notion is well supported in the literature (Xu et al., 1998). The ability of H_2O_2 to kill biofilm bacteria depends upon the concentration of H_2O_2 as well as the concentration of enzymes that degrade it (i.e., catalases and peroxidases); consequently, the dynamic of resistance versus susceptibility can be quite complex. Further, because of the vigorous oxygenic reaction of H_2O_2 with catalases within microorganisms deeply embedded within the biofilm matrix, organisms that were previously in oxygen-limited confines can shift their metabolism within minutes to an aerobic mode of growth, triggering an active oxidative stress response. Similarly, even if organisms at or near the surface of the biofilm perish in the face of H_2O_2, the catalase within these organisms (essentially "sacrificial lambs") can still serve to protect viable organisms that lie beneath. The oxygenic reactions of catalase also aids in the physical dispersion of biofilms via bubbling. It is very clear, however, that *P. aeruginosa* KatA is extremely important in biofilm resistance to H_2O_2 in that a *katA* mutant is hypersusceptible to it (Elkins et al., 1999).

11.2. KatB and AnkB

11.2.1. KatB, a Periplasmic Tetrametric Catalase

The *katB* gene encodes a 228 kDa tetrameric catalase of the Group I catalase family (Klotz et al., 1997). In contrast to KatA activity, KatB activity is detected only when organisms are exposed to H_2O_2 or in a mutant defective in AhpCF activity (see below for details; Ochsner et al., 2000). Despite being a minor fraction of the total wild-type catalase activity (approximately 5%; Brown et al., 1995), a *katB* mutant is still slightly sensitive to H_2O_2 (Brown et al., 1995), although not nearly to the level of a *katA* mutant. However, unlike the *katA* gene, regulation of the *katB* gene is complex. Brown et al. (1995) have shown that exposure of organisms to H_2O_2, the O_2^--generating agent paraquat (which ultimately leads to H_2O_2 production, especially in SOD-containing bacteria), and, to a lesser extent, 50ºC heat shock increases

KatB activity. The major activator of *katB* transcription was found to be OxyR, a member of the LysR family of transcriptional activators, which responds positively to H_2O_2 in *P. aeruginosa*, as in *E. coli* (Ochsner et al., 2000). We will discuss *P. aeruginosa* OxyR in more detail below.

11.2.2. AnkB: An Ankyrin-like Protein

11.2.2.1. Putative Role #1

The protective activity of KatB against H_2O_2 is, in part, dependent upon AnkB, a putative cytoplasmic membrane or periplasmic protein (Howell et al., 2000). The precise location of AnkB was inferred from translational fusions with alkaline phosphatase, which is only active in the periplasmic space (Ma et al., 1999). However, the assumption of the cytoplasmic membrane/periplasmic localization of AnkB may be premature. It was postulated that AnkB anchors KatB at the inner membrane so that its active site is maximally oriented for optimal detoxification of H_2O_2 from the external milieu (Figure 5A). This potential tandem is descriptively tantalizing in a "baseball" sense in that AnkB is the "catcher" and KatB is the "mitt." Such a "battery" could serve to protect sensitive cytoplasmic-membrane enzymes, such as the β-subunit of the *E. coli* F_1,F_0-ATP synthase, from inactivation by exogenous H_2O_2 (Tamarit et al., 1998). However, as yet, we have no definitive proof of a protein–protein interaction between KatB and AnkB. This postulate awaits experimental confirmation or refutation. Below, we offer a second plausible role for AnkB in response to exogenous H_2O_2.

11.2.2.2. Putative Role #2

We caution that the putative role of AnkB vis-à-vis protection against H_2O_2 described in Section 11.2.2.1 may be erroneous. A unique feature of AnkB is that it harbors an RGD (Arg-Gly-Asp) motif that is a recognition sequence for the eukaryotic transmembrane proteins called integrins. Bacterial proteins containing RGD motifs are capable of eliciting a powerful cellular response in human cells by binding integrins. For example, the *Bordetella pertussis*–secreted protein filamentous hemagglutinin (FHA) binds integrin (Relman et al., 1990), an event that ultimately triggers apoptosis. We postulate that AnkB secretion by *P. aeruginosa* in response to phagocyte-generated H_2O_2 leads to binding of AnkB to integrins—akin to the "hunted being the hunter" (Figure 5B).

11.3. KatC

The *katC* gene was discovered fairly recently by scanning the *Pseudomonas* genome (www.pseudomonas.com). Other than *Bacillus subtilis*, *P. aeruginosa* is the only organism that possesses catalases from groups I, II, and III (Klotz et al., 1997). Were it not for the contig data from the genome project, the *katC*

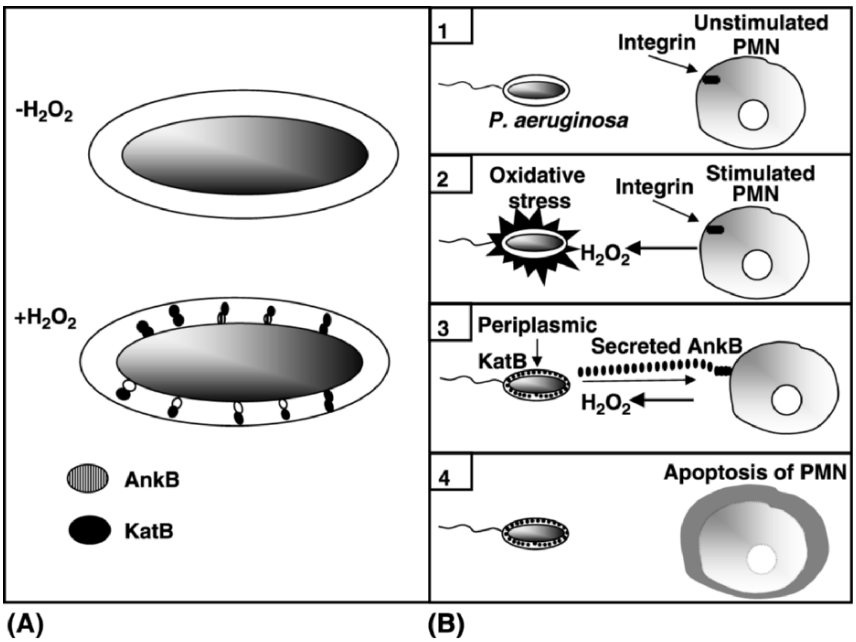

FIGURE 5. (A) The "catcher's mitt" theory of how *Pseudomonas aeruginosa* AnkB (light circles) might position the active site of the periplasmic catalase, KatB (dark circles), for optimal degradation of exogenous H_2O_2. Without AnkB, there is less KatB activity, and increased susceptibility to H_2O_2 (From Howell et al., 2000.) (B) The AnkB "assassin" theory. AnkB may be secreted from *P. aeruginosa* when confronted with H_2O_2 from stimulated human neutrophils (panel 2). The organisms respond by secreting AnkB (panel 3) and deploying KatB to the periplasmic space. Finally AnkB binds to integrin on the neutrophil, triggering apoptosis (panel 4).

gene may have been unrecognized for some time. The reason is that KatC activity provides a paltry 0.1% of the total activity of wild-type organisms (D. J. Hassett, 2004). Although the regulation of the *katA* and *katB* genes is somewhat predictable, with KatA being the "babysitter" and KatB being "911," regulation of the *katC* gene remains a puzzle. An examination of genes adjacent to *katC* revealed little information, except that for the antimicrobial component of the respiratory burst of human neutrophils (Gitzelmann and Bosshard, 1993), a process that involves the production of H_2O_2, we postulated that exposure to glycogen could activate *katC*. This postulate was based upon glycogen catabolic genes in immediate proximity of the *katC* gene (*P. aeruginosa* cannot metabolize glycogen; Schulte et al., 1982). This activation of katC would allow triumvirate of KatA, KatB, and KatC to collaborate in detoxification of H_2O_2, especially in the imposing face of stimulated

neutrophils. Preliminary evidence indicates that glycogen does not activate *katC* based upon native gel activity staining (D. J. Hassett, unpublished data). To date, we know little of the machinery involved in *katC* transcription.

12. Alkyl Hydroperoxide Reductase

Another class of enzymes is involved in protection of *P. aeruginosa* against peroxides: the alkyl hydroperoxide reductases (AHPs). *P. aeruginosa* possesses at least four enzymes of this class that contribute to detoxification of ROOH. These include AhpA, AhpB, AhpCF, and Ohr (Ochsner et al., 2000). Little is known of the function of AhpA or its substrate specifity. However, both AhpB and AhpCF are required for optimal resistance to both H_2O_2 and organic peroxides (Ochsner et al., 2000). Interestingly, an *ahpB* mutant is very sensitive to H_2O_2 but is not especially sensitive to either cumene or *t*-butyl hydroperoxides (Ochsner et al., 2000). The reverse holds true with an *ahpCF* mutant, being more sensitive to organic peroxides and resistant to H_2O_2 (Ochsner et al., 2000).

Another proven AHP of *P. aeruginosa* is the Ohr. The *ohr* gene product is required for resistance to organic peroxides but not to H_2O_2 or the redox-cycling agent paraquat. An *ohr*-null mutant was hypersusceptible to OHPs in disk inhibition assays and showed enhanced killing by OHPs in liquid culture (Ochsner et al., 2001). The *ohr* gene product was demonstrated to contribute to the decomposition of OHPs. Transcription was induced up to 15-fold upon exposure to OHPs, and this induction was independent of OxyR. Somewhat enhanced *ohr* transcriptional activity was detected in mutant strains defective in *ohr*, *ahpC*, and *oxyR*, and this phenotype correlated with hypersusceptibility to OHPs, suggesting that the *ohr* and *ahpC* gene products may have overlapping or compensatory functions. A single transcriptional start site for *ohr* was determined, and *ohr* transcripts were abundant in cells treated with a sublethal dose of OHPs but not in cells treated with paraquat. An 84 bp portion upstream of the *ohr* mRNA start site was sufficient for *ohr* induction by OHPs. Thus, the *ohr* gene appears to encode an antioxidant enzyme that is not part of the OxyR regulon, yet is specifically induced by OHPs. At present neither the biological sources nor the targets of OHPs are known.

13. OxyR

Although the majority of genes of the OxyR regulon in *P. aeruginosa* are unknown, several candidate genes have recently been discovered using GeneChip microarrays to analyze the response of wild-type organisms to H_2O_2 (Palma et al., 2004). As discussed above, OxyR controls expression of the *katB-ankB* operon, the *ahpB-tdr* operon, and *ahpCF* (Ochsner et al., 2000). The *ahpB* and *tdr* genes encode a cytosolic thiol-specific peroxidase

and a thioredoxin reductase, respectively. This makes strategic sense in that KatB is deployed to the periplasm. Thus, an *oxyR* mutant cannot produce KatB or AnkB unless other, as yet unknown, compensatory mechanisms are available. Still, despite possessing near-wild-type catalase activity from KatA, an *oxyR* mutant is dramatically sensitive to H_2O_2, even more so than a *katA katB* double mutant. The *recG* locus is immediately downstream and in a small operon with *oxyR*, but a *recG* mutant does not exhibit the H_2O_2-sensitive phenotype of the *oxyR* mutant. Therefore, an unidentified member of the regulon is critical for H_2O_2 resistance. We have solid evidence that AhpB and KatB-AnkB are the two major players involved in protection of sensitive inner-membrane proteins that include the F_1,F_0-ATPase.

Perhaps the most novel phenotype of the *oxyR* mutant is its inability to grow in liquid or solidified media when diluted to less than 10^7 CFU/ml (Hassett et al., 2000). When overnight aerobic cultures of wild-type and *oxyR* mutant bacteria are serially diluted in sterile spent culture supernatant, both organisms will form isolated colonies when spotted as a 20 µl suspension on L-agar. However, when the *oxyR* mutant is diluted in L-broth, bacteria fail to grow on L-agar when plated at a density of 10^7 CFU/ml or less (Figure 6).

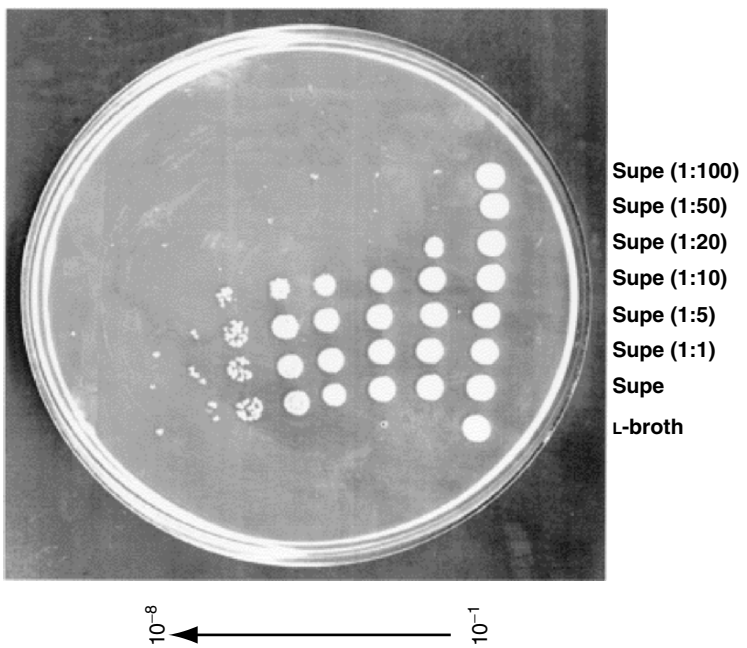

FIGURE 6. Luria (L) agar plate containing 20 µl spots of serially diluted *oxyR* bacteria. (From Hassett et al., 2000.) Note that sterile ultrasupernatants of stationary phase cultures that contain the major catalase, KatA, protect *oxyR* mutant bacteria from serial dilution on aerobic peroxidogenic L-agar plates. However, once the supernatants are diluted beyond a factor of 10-fold there is little or no protection of the *oxyR* mutant.

These mutants as well as wild-type organisms grew when they were serially diluted in sterile supernatant from cultures of wild-type organisms. Boiling of the wild-type spent culture supernatant eliminated its protective component. This suggested that a heat-labile factor was present in the spent culture supernatant that was essential for plating efficiency and growth in broth of the *oxyR* mutant. This component was found to be KatA. L-broth itself contains autoxidizable components that are capable of generating approximately 1.2 μM H_2O_2 per minute at room temperature (Hassett et al., 2000). Evidently, this amount of H_2O_2 is sufficient to kill approximately 10^7 *oxyR* mutant bacteria.

14. Glucose-6-Phosphate Dehydrogenase

G6PDH of *P. aeruginosa* converts glucose-6-phosphate to 6-phosphogluconolactone. The enzyme utilizes both NAD^+ and $NADP^+$ as cofactor. Bacteria that lack G6PDH are more sensitive to paraquat (Ma et al., 1998). The reason for this sensitivity may be that the reducing power of NADPH is critical for the activity of glutathione reductase.

15. The Phagocytic Respiratory Burst: Two Important Pathogens that Resist Killing

15.1. Salmonella typhimurium *Oxidative Stress Systems*

Virulent *S. typhimurium* invades, survives, and replicates within host macrophages. To do so, it must either avoid or tolerate the O_2- and H_2O_2 that is generated by the phagocytic oxidative burst. Part of its strategy is to secrete virulence factors that block the delivery of the NADPH oxidase to the phagosome. This tactic is only partly successful, however, as infected macrophages continue to generate detectable amounts of oxidants. For this reason investigators have probed to determine the roles that antioxidant enzymes play in *Salmonella* virulence.

In general, *Salmonella* expresses the same set of antioxidant proteins as its close relative, *E. coli*. These have been screened for their involvement in pathogenesis. Interestingly, neither the transcriptional regulator SoxS, which governs transcription of the *S. typhimurium* superoxide response, nor MnSOD is required for full virulence in mice (Fang et al., 1997)—perhaps because macrophage-generated superoxide cannot penetrate the lipid membrane into the bacterial cell. G6PDH, however, is important—unless the respiratory burst is blocked (Lundberg et al., 1999). Mutations in the *oxyR* and *katG* genes do not affect virulence (Papp-Szabo et al., 1994), but elimination of Dps does (Halsey et al., 2004). It may be that H_2O_2 titers within the phagosome are high enough that scavenging enzymes have little impact, whereas Dps, which reduces the free-iron level, can effectively protect DNA.

None of those genes uniquely serve *Salmonella* in its role as an intracellular pathogen. However, the story appears to be different with the periplasmic SOD activity. Cu,Zn-SOD was found by De Groote et al. (1997) to be required for resistance to both O_2- and NO. The activity was also required for survival during systemic salmonellosis (Farrant et al., 1997). A reasonable thought is that periplasmic SOD protects components of the periplasm from the O_2^- that is generated by the NADPH oxidase. Further study revealed that *S. typhimurium* actually possesses two periplasmic SODs, designated SodCI and SodCII (Fang et al., 1999). The SodCII enzyme is that which most resembles the Cu,Zn-SOD of *E. coli*, in that it is highly expressed in stationary phase (Fang et al., 1999) and under control of the stationary phase sigma factor, RpoS (Fang et al., 1999). In contrast, the *sodC1* gene product is encoded by a lysogenic bacteriophage. To date, all *Salmonella* strains harbor the *sodCII* gene, yet only the most pathogenic strains carry the phage-associated *sodCI* genes. In mouse infection models SodCI appeared to be particularly important for virulence (Klotz et al., 1997). This enzyme appears to be tethered somehow within the periplasm even when the outer membrane is disrupted, an unusual attribute that may somehow contribute to its role during infection.

16. Mycobacterium Tuberculosis Oxidative Stress Systems

M. tuberculosis is an obligate intracellular pathogen that actually modifies the architecture of the alveolar macrophage once the organism is engulfed. Parallels with *Salmonella* are apparent. *M. tuberculosis*, like *S. typhimurium*, inhibits fusion of the phagosome in which it initially resides, with lysosomes, an event that allows it to survive and propagate within such cells. Recently, however, ATP was found to promote phagosome–lysosome fusion in *M. tuberculosis*–infected macrophages in a calcium-dependent fashion (Fairbairn et al., 2001; Kusner and Barton, 2001). This represents a major advance in the potential treatment of patients infected with *M. tuberculosis*. As with *Salmonella*, extracytosolic SOD activity is important: mutants that lack these enzymes are hypersensitive to oxidative stress agents and phagocytic killing, and are altered in virulence properties. For example, the Cu,Zn-SOD of *M. tuberculosis* enhances survival of the organism when inside macrophages (Piddington et al., 2001). Strikingly, the secreted Fe-SOD (also known as SodA) also contributes to the virulence of *M. tuberculosis* by paralyzing the innate host immune response (Edwards et al., 2001). Secretion of both Fe-SOD and KatG (a trifunctional catalase-peroxidase-peroxynitratase; Wengenack et al., 1999) is SecA2-dependent (Braunstein et al., 2003). In contrast to SOD, KatG has been shown to be required for optimal disposal of peroxides generated by macrophages, but is not required for resistance to reactive nitrogen intermediates (Ng et al., 2004).

The most interesting role for KatG in the context of *M. tuberculosis* long-term survival is its role in activating isoniazid (INH). INH is a front-line antibiotic that paralyzes the synthesis of mycolic acids, a cell wall component that is essential for survival of *M. tuberculosis*. However, INH is only a pro-drug, because it requires an "activation" step once it enters the tubercle bacillus. This activation is mediated by the peroxidatic activity of KatG (Zhang et al., 1992). Thus, organisms that possess wild-type KatG are susceptible to INH treatment, while those without it or with KatG (S315T) are resistant (Wengenack and Rusnak, 2001).

17. Conclusions

Bacteria appear to have at least two categories of defenses against oxidants: basal defenses, dominated by constitutive scavenging enzymes, which are necessary to forestall toxicity from endogenous O_2- and H_2O_2; and inducible defenses, which respond and cope with excessive levels of these species that arise from redox-cycling drugs or diffusive entry of exogenous H_2O_2. There is substantial overlap among defensive strategies: *E. coli*, *P. aeruginosa*, *S. typhimurium*, and *M. tuberculosis* employ scavenging enzymes, iron sequestration, and oxidant-resistant isozymes in order to endure stress. However, the titers and controls of these enzymes can differ substantially and are dependent on mode of metabolism, nutrient availability, and competition. These contrasts are likely to reflect differences in the intensity and circumstances of oxidative stress, as dictated by their obviously distinct lifestyles and habitats. Construction of a fully integrated view of oxidative physiology will, therefore, require that investigators broaden their efforts to incorporate considerations of the ecology and pathogenic breadth of these organisms.

Questions to Consider

1. Lactic acid bacteria deliberately generate millimolar concentrations of H_2O_2, which suppresses the growth of competitors. But these organisms have plenty of iron in them–so how are they able to tolerate endogenously generated H_2O_2?

2. How does iron get trafficked through cells?

Aside from its reactivity with H_2O_2, iron is sticky and probably needs a carrier to avoid glomming onto every protein, nucleic acid, and membrane it encounters.

3. Do physiological concentrations of oxidants oxidize cysteine residues on proteins?

OxyR and Ahp have very reactive sulfhydryls–because they evolved to be that way–and it seems likely that there are similarly oxidizable thiols on proteins whose function would be compromised by oxidation. However, I don't think there is any good evidence that moderate concentrations of H_2O_2 (or superoxide) oxidize protein cysteines.

4. What are the roles of the OxyR-and SoxRS-inducible genes whose functions are not yet known?

5. Borrelia, which has no Fe enzymes, nevertheless has an SOD. What kind of damage is it preventing?

6. What periplasmic biomolecules does periplasmic CuZnSOD protect?

We know nothing about damage in this compartment.

7. Why are some organisms with vast reservoirs of antioxidant enzymatic machinery in the form of SODs, catalase, and alkyl hydroperoxide reductases, remarkably more sensitive to oxidative stress agents than organisms harboring a comparable paltry less antioxidant armamentarium?

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Chapter 15

Bacterial Biowarfare Agents

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Historical Landmarks

1346 Mongols catapult plague-infected corpses over the walls into Kaffa , with the intent of causing a plague epidemic upon the Genoan enemy (Derbes, 1966).

1767 During the French and Indian War, British forces in North America give blankets used by smallpox patients to the Native Americans (Christopher et al., 1997).

1917 Germans use anthrax and glanders (*Burkholderia mallei*) to infect livestock and animal feed for export to the Allied Forces (Christopher et al., 1997).

1937 Japan creates "Unit 731," a BW (Biowarfare) research facility in Manchuria, where experimental infections were carried out on Chinese prisoners. More than 10,000 people die after exposure to plague, anthrax, tularemia, syphilis, and other agents. It is believed that the facility also had millions of rats infected with fleas carrying *Yersinia pestis* (Girdwood, 1985; Harris, 1992).

1939 Japan poisons Soviet water supply with intestinal pathogens at Mongolian border (Nomonhan incident) (Williams and Wallace, 1989).

1940 Japan drops rice and wheat mixed with plague-carrying fleas over China and Manchuria (Williams and Wallace, 1989).

1942 The U.S. begins biological weapons program and chooses Camp Detrick, Frederick, Maryland, as its research and development site. Research efforts initially concentrated on the use of anthrax and botulinum toxin as bioweapons (Christopher et al., 1997).

1943 England tests anthrax bombs to kill sheep on Gruinard Island ("Anthrax Island") off the coast of Scotland. Viable anthrax spores were still found on the island 40 years later (Manchee et al., 1982).

1979 Outbreak of pulmonary anthrax in Sverdlovsk, U.S.S.R. caused by an accidental release of anthrax spores from a Soviet military microbiological facility. Hundreds are exposed and at least 67 die (Meselson et al., 1994).

1984 Outbreak of salmonellosis in Oregon, U.S., after members of the Rajneesh cult intentionally contaminate salad bars with *Salmonella typhimurium* (Torok et al., 1997).

1985 Iraq develops biological weapons including anthrax, botulinum toxin, and aflatoxin (Christopher et al., 1997).

1993 Members of the Japanese cult of Aum Shinrikyo attempt an aerosolized release of anthrax from the tops of buildings in Tokyo (Smithson, 2000).

1996 Outbreak of *Shigella dysenteriae* in Texas, U.S. after workers ingest food that was intentionally contaminated with this bacteria (Kolavic et al., 1997).

2001 Release of anthrax spores through the U.S. mail. A total of 22 confirmed cases of bioterrorism-related anthrax; 5 people die (Fennelly et al., 2004; Jernigan et al., 2001).

1. Biowarfare Agents and Historical Perspective

Bioterrorism can be defined as the dissemination of biological agents with the intention to induce disease and spread fear and panic. The release of microbial pathogens (such as *Bacillus anthracis* or *Yersinia pestis*) or biological toxins (such as botulinum toxin or ricin) can result in serious morbidity and mortality and perhaps, more importantly, it can cause great disruption to society, massive public health crises, and tremendous impact on worldwide economy.

According to the U.S. Centers for Disease Control and Prevention (CDC), potential biological biowarfare agents are classified into three categories: A, B, and C (Table 1).

> Category A agents are the highest-priority pathogens. They pose the greatest risk to national security because they (a) can be easily disseminated or transmitted from person to person, (b) result in high mortality rates and have the potential for major public health impact, (c) might cause public panic and social disruption, and (d) require special action for public health preparedness. Category B agents include pathogens that are moderately easy to disseminate, result in moderate morbidity rates and low mortality rates, and require specifically enhanced diagnostic capacity. Category C agents include emerging pathogens, to which the general population lacks immunity, and that could be engineered for mass dissemination because of ready availability, ease of production, ease of dissemination, potential for high morbidity and mortality, and major public health impact. (CDC, 2005)

In this chapter, we present an overview of the most important aspects of virulence, pathogenicity, interactions with the host, and prevention of bacterial pathogens. These organisms are potential bioweapons because their intentional release in the environment can induce severe, debilitating, and potentially lethal infections. Our discussion will focus on *B. anthracis*, *Y. pestis*, and *Francisella tularensis*, which represent the most currently investigated bacterial pathogens in category A. The use of botulinum toxin as a bioweapon would cause "inhalational botulism," which in essence is an "intoxication" rather than an infection, and therefore will not be discussed here.

The use of microbial pathogens as potential weapons of terrorism dates to ancient times. A recount of the use of bacterial bioweapons beginning with the known earlier instances to the most current events is given in the Historical Landmarks. Although the veracity and exact epidemiological and bacteriological data from some of the earlier reported events are impossible to confirm, this historical review reveals that humans have had and continue

TABLE 1. CDC classification of bioterrorism agents/diseases.

	Bacteria	Toxin	Virus
Category A	*Bacillus anthracis* *Yersinia pestis* *Francisella tularensis*	Botulinum toxin (from *Clostridium botulinum*)	Variola major (smallpox) Filovirus (e.g., Ebola, Marburg) Arenavirus (e.g., Lassa, Machupo)
Category B	*Brucella* species *Burkholderia mallei* *Burkholderia pseudomallei* *Chlamydia psittaci* *Coxiella burnetii* *Rickettsia prowazekii* *Salmonella, Shigella* *Escherichia coli* O157:H7 *Vibrio cholerae*	Epsilon toxin (from *Clostridium perfringens*) Staphylococcal enterotoxin B (SEB) Ricin	Viral encephalitis—alphavirus such as: Venezuelan equine encephalitis Western equine encephalitis Eastern equine encephalitis
Category C			Nipah virus Hantavirus Severe acute respiratory syndrome (SARS)

to have a keen interest in the development and malicious use of biological weapons.

The terrorist events of September 11, 2001 in the U.S., followed by the dissemination of anthrax spores via the postal service, materialized the vulnerability of our society to these kinds of attacks and underscored the urgency for developing new strategies of countermeasurement, detection, diagnosis, treatment, and prevention of infections caused by microbial biowarfare agents. In this regard, the scientific community is in the presence of great challenges and unprecedented opportunities. The outcome of the renewed scientific initiatives on bioterrorism will undoubtedly be a more comprehensive understanding of the pathogenesis and host interactions of these pathogens, as well as the identification of new targets for the development of efficacious prophylaxis and therapeutics. This information will not be limited to bacterial bioweapons, but will be broadly applicable to other important human microbial pathogens.

2. Anthrax

2.1. Introduction

Anthrax is caused by the Gram-positive bacterium *B. anthracis*. In nature, anthrax is a disease that herbivores or other mammals acquire after contact with *B. anthracis* spores present in soil. Human disease results from contact

with infected animals or contaminated animal products, or after exposure to accidentally or intentionally released spores. Anthrax was originally called Woolsorter's disease because it occurred most frequently in people who worked in animal textile mills. *B. anthracis* derives its name from the morphology of the microscopic bacterium (baculum, Latin for rod), and the dry, black appearance of cutaneous anthrax lesions (anthrakis, Greek for coal).

Beginning in 1972, nations around the globe signed a treaty at the Biological and Toxin Weapons Convention banning the research of biological weapons for offensive purposes. Despite this written agreement, many nations secretly continued to develop bioweapons (Cole, 1996). In April and May of 1979 a military facility in the northwest region of Sverdlovsk, U.S.S.R. (now Ekaterinburg), accidentally released *B. anthracis* spores into the air. Prevailing winds carried the spores into the nearby town resulting in 96 reported cases of pulmonary anthrax and at least 64 deaths (Meselson et al., 1994). Russian officials initially claimed that most of the cases were gastrointestinal anthrax acquired as a result of consumption of contaminated meat; however, subsequent laboratory analyses of patient samples and epidemiological studies concluded that the infections resulted from inhalation of aerosolized spores originating from the nearby military base (Bezdenezhnykh and Nikiforov, 1980; Meselson et al., 1994). This was the first known incident of human infection resulting from weaponized *B. anthracis* spores. Most recently, in October 2001, 22 individuals became infected with anthrax after the intentional release of spores through the U.S. mail. As a result, 11 confirmed cases of inhalation anthrax and 11 confirmed or suspected cases of cutaneous anthrax were diagnosed. There were 5 fatalities, and prophylactic antibiotics were recommended to approximately 10,000 people (Shepard et al., 2002). The fifth and final pulmonary anthrax fatality occurred months after the initial cases in a 94-year-old Connecticut woman who could not be directly linked to a known infectious source (Jernigan et al., 2001). Since this chapter deals specifically with agents of biowarfare capability, the majority of this section will focus on pulmonary anthrax.

2.2. Pathogenesis

Three distinct clinical forms of anthrax disease can develop in humans depending on the mode of infection (Figure 1). Cutaneous anthrax is the most common type of infection, usually acquired by people who are in frequent contact with infected animals or animal products. Disease results after entry of infectious spores into abrasions or cuts in the skin. Initially the infection appears as a small painless lesion that resembles an insect bite. The lesion is often pruritic, and develops into a larger inflamed papule. The raised area results from local fluid accumulation, caused at least in part by the action of anthrax toxins. As the tissue becomes necrotic, a dry black eschar forms and

eventually dries and sloughs off a few weeks after formation (Kalamas, 2004). Antibiotic treatment confers a 99% survival rate for patients diagnosed with cutaneous anthrax (Karwa et al., 2003). Dissemination to the bloodstream can occur in the absence of treatment, but is very rare if treatment is sought promptly. Gastrointestinal anthrax results from the ingestion of meat contaminated with *B. anthracis* spores. The infection is characterized by the presence of ulcers, massive edema, and necrosis of the intestinal mucosa. This is the rarest form of anthrax and, although treatable when diagnosed promptly, death can occur due to intestinal perforation and systemic dissemination of the toxins.

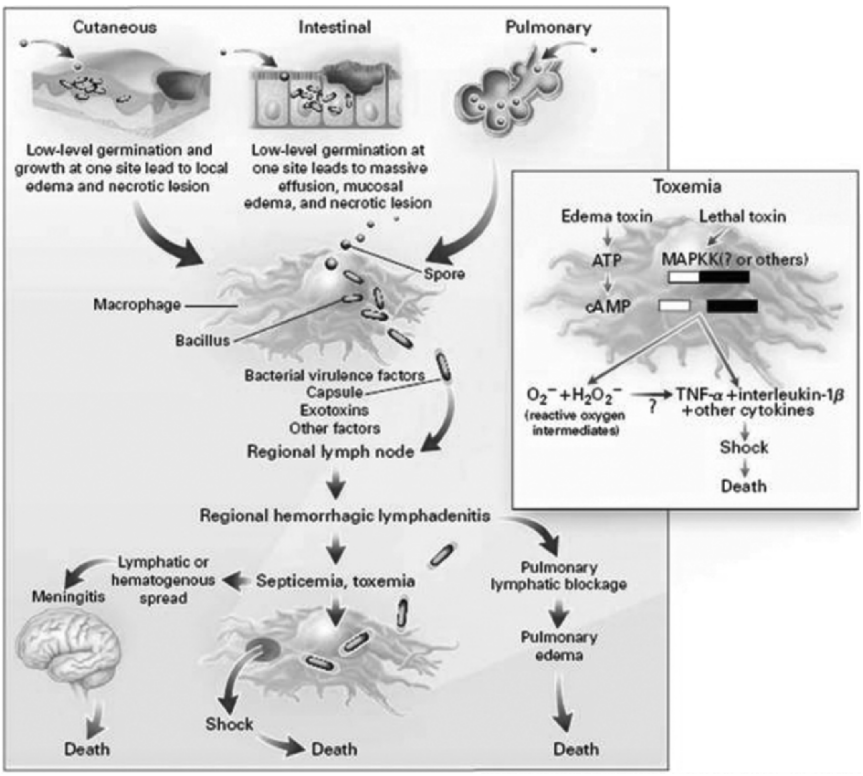

FIGURE 1. *Bacillus anthracis* endospores reach a primary site in the subcutaneous layer, gastrointestinal mucosa, or alveolar spaces. For cutaneous and gastrointestinal anthrax, low-level germination occurs at the primary site, leading to local edema, necrosis and, occasionally, to systemic spread. In cases of pulmonary anthrax, peribronchial hemorrhagic lymphadenitis blocks pulmonary lymphatic drainage, leading to pulmonary edema. Death results from septicemia, toxemia, or pulmonary complications. The action of Edema toxin and Lethal toxin are shown on the right. (Reproduced with permission from Dixon et al., 1999.)

Pulmonary anthrax is the most serious manifestation of the disease and results from inhalation of aerosolized *B. anthracis* spores. To induce a productive infection, the spores must reach the alveolar spaces of the lung where resident macrophages phagocytize them and germination occurs. To reach the alveolar spaces and avoid the mechanical innate barriers of the host, spores must be between 1μm and 5μm in diameter (Hatch, 1961). This requirement represents one of the major technological hurdles for the "weaponization" of *B. anthracis* and may be one of the reasons why weaponized anthrax can only be produced with considerable financial and technological resources. Pulmonary anthrax is the most likely outcome in instances of biological warfare, since once the microscopic spores are released in the environment they can be rapidly disseminated and inhaled by humans (Jernigan et al., 2001; Meselson et al., 1994). Although pulmonary anthrax is highly fatal and easily contracted from aerosolized spores, it has not been shown to be transmissible from person to person and is thus limited in its ability to cause mass casualties. This is in contrast to pneumonic plague, which can be acquired after inhalation of aerosolized infectious bacteria and subsequently disseminated from infected individuals to close contacts.

Following inhalation, alveolar macrophages ingest the dormant spores and germination is triggered by a series of host-and/or bacteria-dependent events, most of which are not yet completely understood (Bergman et al., 2005; Guidi-Rontani et al., 1999; Weiner and Hanna, 2003). From studies in a murine model it was determined that germination begins within 1 h of intranasal or intratracheal inoculation, and after 5 h vegetative bacilli can be found in regional lymph nodes (Lyons et al., 2004). Some of the spore-containing macrophages migrate to the peribronchial and mediastinal lymph nodes where vegetative bacilli continue to replicate and induce initial hemorrhagic mediastinitis (Abramova et al., 1993). Once outside macrophages, the bacilli multiply rapidly, and synthesize copious amounts of toxins that are hematogenously disseminated, leading to the clinical manifestations and the mortality associated with the disease. Pulmonary anthrax has been described as a biphasic illness in which the initial symptoms occur within days of infection (Abramova et al., 1993; Brookmeyer et al., 2005). Symptoms in the first phase are nondistinct, resemble a mild flu infection, and include fever, chills, headache, cough, and malaise. The second phase is a fulminant disease characterized by chest pain, and significant respiratory distress evident by severe cough, shortness of breath, and harsh, labored respiration (Kalamas, 2004). In the absence of treatment, death often follows 2–3 days after onset of the severe symptoms. Characteristic clinical presentations of pulmonary anthrax include pleural effusions and mediastinal widening observed on chest X-radiograms (Dixon et al., 1999; Jernigan et al., 2001). Death apparently results from multiorgan failure similar to systemic shock.

Pathology evaluation of tissues obtained from the pulmonary anthrax victims of the 2001 bioterrorist attack identified common abnormalities such as mediastinal edema, necrotizing mediastinitis, liver hypoxia, lymphadenopathy,

and hemorrhagic bilateral pleural effusions. Cultures of nasal swabs, serosanguinous fluid, blood, and cerebral spinal fluid were not always positive for *B. anthracis*, particularly in early stages of the disease. In fact, in several cases, samples did not test positive for *B. anthracis* until postmortem analysis. Confirmation of *B. anthracis* DNA by PCR was often successful and represented a more sensitive test than bacterial culture or Gram stain analyses (Jernigan et al., 2001).

2.3. Virulence Determinants

The ability of *B. anthracis* to induce a fulminant disease has been primarily attributed to the presence of an antiphagocytic capsule and the production of two toxins: anthrax lethal toxin (LeTx) and anthrax edema toxin (EdTx). A number of genes important in the pathogenesis of *B. anthracis* have been identified, but the most extensively studied are found on two virulence plasmids called pXO1 and pXO2 (Green et al., 1985). Plasmid pXO1, originally named pBA1, is 184 kb and contains the genes necessary for synthesis of the three components of the anthrax toxins. Also present on pXO1 is the *atxA* gene, which encodes a transactivator protein involved in the regulation of many *B. anthracis* genes (Bourgogne et al., 2003; Dai et al., 1995; Uchida et al., 1993). Experimental evidence indicates that the primary determinant of anthrax virulence is the presence of lethal factor (LF) and protective antigen (PA) which together form LeTx (Beall et al., 1962; Cataldi et al., 1990; Ezzell et al., 1984; Klimpel et al., 1994; Park et al., 2002; Pezard et al., 1991; Pitt et al., 2001; Reuveny et al., 2001). The other virulence plasmid, pXO2, contains the genes responsible for synthesis of the bacterial capsule (Green et al., 1985).

2.3.1. Capsule

Wild-type *B. anthracis* synthesizes a unique proteinaceous capsule composed exclusively of gamma-linked D-glutamic acid residues. Although the exact role of the capsule in infection has not been elucidated, it is known to be effective at preventing phagocytosis by macrophages and is also necessary for bacterial systemic dissemination (Drysdale et al., 2005; Makino et al., 1989). The enzymes responsible for capsule synthesis are encoded by the genes *capA*, *capB*, *capC*, and *capD* located on the 96 kb pXO2 virulence plasmid (Makino et al., 1989, 2002). Collectively these four genes make up the capBCAD operon. A strain with the capBCAD operon deleted is highly attenuated in a murine model of inhalation anthrax (Drysdale et al., 2005). Since the capsular material has been shown to be poorly immunogenic, it is believed that the capsule is necessary to avoid recognition by antibodies *in vivo*, and therefore the lack of capsule can result in accelerated clearance by the host (Leonard and Thorne, 1961). It has been postulated that pXO2 may carry other genes important for the virulence of the organism. Transposon mutagenesis of

pXO2 produced mutants that synthesized wild-type levels of capsule *in vitro* but exhibited reduced virulence when injected subcutaneously in mice (Welkos, 1991).

2.3.2. Toxins

Virulence of *B. anthracis* is complemented by the action of two toxins: LeTx and EdTx. EdTx and LeTx conform to the classical model of bacterial A-B toxins, since both are composed of a receptor-binding B subunit and a catalytically active A subunit. Unlike most binary toxins, the two components are not covalently linked, but instead exist as three separate proteins, before binding to the host cell. Initial binding to the host cell receptor is accomplished by the 83 kDa protective antigen (PA) protein encoded by the *pagA* gene located on virulence plasmid pXO1 (Vodkin and Leppla, 1983). The recently identified receptors for anthrax toxin are the tumor endothelial marker (TEM8) (Bradley et al., 2001) and the capillary morphogenesis protein2 (CMG2) (Scobie et al., 2003). These receptors are present in a broad range of tissues and contain a common extracellular von Willebrand factor type A domain, also called integrin-like domain (I-domain), which constitutes the PA binding site. The crystal structure of the interaction between PA and its receptors has been described in detail (Santelli et al., 2004).

After binding to the host cell receptor, a furin-like endoprotease cleaves the PA monomer at residue R167 releasing a small, N-terminal, 20 kDa fragment (Klimpel et al., 1992; Singh et al., 1989). The larger peptide (63 kDa) left on the cell surface, assembles into heptamers that can bind to either of the two A subunits: edema factor (EF) or lethal factor (LF); or some combination of the two (Pimental et al., 2004), producing a functional toxin molecule. PA-deficient strains of *B. anthracis* are avirulent in animal models attesting to the importance of the binding subunit in toxin-mediated pathogenesis (Cataldi et al., 1990; Pezard et al., 1991). When a molecule of LF attaches to a bound, activated molecule of PA, the complex is referred to as LeTx (lethal toxin). If a molecule of EF binds, the resulting complex is called EdTx (edema toxin). *B. anthracis* is unique in this respect since both catalytic proteins share the same binding subunit. Current evidence suggests that a maximum of three ligand molecules can bind per each heptamer, although it is not known if both EF and LF can bind simultaneously to one heptamer (Cunningham et al., 2002; Mogridge et al., 2002; Mourez, 2004). Following binding of the EF or LF subunits, the cell engulfs the PA/LF/EF complex via receptor-mediated endocytosis (Gordon et al., 1988). Each member of the PA heptamer then inserts a β-barrel hairpin loop into the vesicle membrane forming a pore that allows the translocation of the catalytic subunit(s) to the cytosol where they initiate their toxic effects (Nassi et al., 2002). Acidification of the endosome has been shown to be essential for the toxic effects *in vitro* suggesting that formation of the pore and/or translocation of the A subunit is pH-dependent (Friedlander, 1986; Milne et al., 1994). The insertion of PA

into the membrane seems to occur soon after the toxin complex is internalized, while the delivery of LF or EF to the cytosol is a later event in the endocytic pathway (Abrami et al., 2004) (Figure 2).

Whether LeTx is injected directly in purified form or synthesized by vegetative bacilli, it is believed to be the primary cause of mortality in animal models (Beall et al., 1962; Ezzell et al., 1984; Pezard et al., 1991). The catalytically active component of LeTx is the LF protein encoded by the *lef* gene. Work by Klimpel et. al. identified this 776-amino acid protein as a metalloprotease with essential zinc-binding histidine residues at positions H686 and H690 and a glutamate residue at position E687 required for LF's catalytic activity (Duesbery et al., 1998; Klimpel et al., 1994). These residues make up the HExxH consensus sequence, a motif that is common to many metalloproteases (Klimpel et al., 1994). It is likely that the PA subunit of LeTx exists

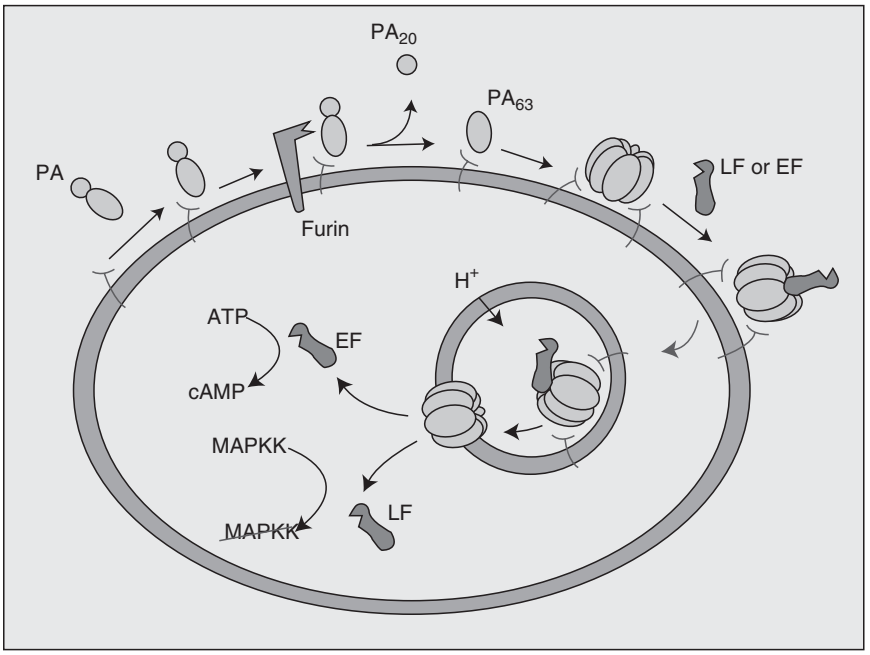

FIGURE 2. Anthrax toxin action. Protective antigen binds to a cellular receptor and is activated by a furin-like protease. The PA_{63} fragment oligomerizes on the cell surface to form a ring-shaped heptamer, which binds lethal factor (LF), edema factor (EF), or both. The complexes are endocytosed and trafficked to an acidic compartment. Subsequently, the heptamer inserts into the membrane and forms a transmembrane pathway for transfer of EF and LF to the cytosol, where they catalyze their respective reactions. PA, protective antigen; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate. (Reproduced with permission from Starnbach and Collier, 2003.)

solely for the purposes of binding and entry since purified LF protein is fully capable of performing its catalytic activity when delivered directly into the cytosol of susceptible cells (Duesbery et al., 1998; Friedlander et al., 1993a).

LF targets members of the mitogen-activated protein kinase kinase (MAPKK) family of signaling proteins (Fig. 1). Within this family, MAPKK1, MAPKK2, and MAPKK3 (aka Mek 1–3) have been shown to be specific targets of LF proteolytic activity (Duesbery et al., 1998; Pellizzari et al., 1999; Vitale et al., 1998). Cleavage of these molecules near their terminus prevents interaction with their respective targets, thus altering vital intracellular signaling functions such as those involved in cell proliferation, cell differentiation, and cell survival (Duesbery et al., 1998). Although cleavage of Mek 1–3 is likely to be an advantage in suppression of the host immune response, it is unknown whether this particular function of LeTx is directly involved in the mortality associated with the disease. It is also possible that LF acts on other intracellular targets; however, no other substrates outside of the MAPKK family of signal transduction proteins have been identified.

Among the downstream effects of MAPKK are the impairment of dendritic cells in their ability to prime CD4+ T cells, inhibition of pro-inflammatory cytokine production, and macrophage lysis via inhibition of the p38 MAPK signaling pathway (Agrawal et al., 2003; Park et al., 2002; Pellizzari et al., 1999). In 1986 Friedlander observed that LeTx could induce rapid lysis of certain murine macrophages, leading to the release of pro-inflammatory cytokines (Friedlander, 1986). This observation gave rise to the hypothesis that macrophages play an important role in the progression of the disease and that a cascade of macrophage-derived pro-inflammatory cytokines was responsible for the shock-like effects observed in toxin-challenged animals. However, this theory is currently the subject of intense debate, because it is now known that humans or certain animals that are very sensitive to both anthrax infection and anthrax toxin have macrophages that are resistant to the action of the toxin *in vivo* (Cui et al., 2004; Friedlander et al., 1993a; Moayeri et al., 2003; Roberts et al., 1998). Therefore, LeTx-mediated death cannot be explained solely as a result of macrophage cytotoxicity. The observations that (1) LF negative strains of *B. anthracis* are avirulent in animals and (2) catalytically inactive LF mutants fail to cleave MAPKK members *in vitro* suggest that a strong correlation exists between the disruption of intracellular signaling events by LF and the mortality caused by LeTx (Duesbery et al., 1998; Pezard et al., 1991).

Despite the growing amount of information on the molecular mechanisms of LF and the multiple effects of the toxin observed *in vitro* and *in vivo*, the specific series of events that lead from LeTx exposure to sudden death remain unknown. In animals and humans, death has been associated with liver hypoxia and respiratory failure (Borio et al., 2001; Moayeri et al., 2003). Strong evidence arguing against the inflammatory cytokine-induced shock theory was presented in experiments in which TNF-receptor knockout and

wild-type mice were equally susceptible to infection after intraperitoneal injection of anthrax spores (Kalns et al., 2002). There are numerous conflicting reports regarding whether the inflammatory cytokine production is induced, suppressed, or unaffected after LeTx exposure (Cui et al., 2004; Erwin et al., 2001; Hanna, 1999; Hanna et al., 1993; Popov et al., 2004).

B. anthracis is one of several species of bacteria that produces adenylate cyclase toxin (Ahuja et al., 2004). Edema factor encoded by the *cya* gene is a 767-amino acid protein that functions as a calmodulin-dependent adenylate cyclase (Fig. 1) (Leppla, 1982, 1984; Robertson et al., 1988). Direct injection of purified EdTx into animals results in local fluid accumulation and swelling (Stanley and Smith, 1961). It has been suggested that an important function of EdTx may be the functional suppression of neutrophils. This was demonstrated when EdTx-treated neutrophils showed a decreased ability to phagocytose killed and opsonized *B. anthracis* (O'Brien et al., 1985). Theoretically, this would aid in survival and spread of the bacteria, and in conjunction with LeTx would disable the first line of protection against infection. The contribution of EdTx to systemic anthrax is not known, but it is assumed that the most significant role for EdTx is in the cutaneous form of the disease. A possible synergistic effect of the two toxins on the host immune system has also been proposed (Pezard et al., 1991; Smith and Stoner, 1967).

2.3.3. Gene Regulation

B. anthracis relies on a combination of environmental signals and responder molecules to control expression of genes critical to survival and virulence. Studies of capsule expression in a $pXO1^{-}$ $pXO2^{+}$ strain revealed that *acpA*, a gene present on pXO2 was essential for the transcription of the *cap* operon and expression of the encapsulated phenotype (Vietri et al., 1995).

The transcriptional transactivator gene, *atxA*, has been shown to regulate expression of the three toxin components of *B. anthracis* and to play a role in expression of the bacterial capsule (Uchida et al., 1993, 1997). Consistent with the importance of *atxA* in toxin gene expression is the observation that an atxA-null mutant is avirulent in mice (Dai et al., 1995). Shortly after *atxA* was identified, work by a separate group noted that temperature and CO_2 levels also influence toxin gene expression (Sirard et al., 1994). This is not surprising since germination of spores in the lungs of susceptible individuals is closely followed by toxin production. It is very likely that bacteria use the change in temperature and CO_2 levels as stimulus to turn on molecular switches that upregulate genes necessary for vegetative growth and protection against immune effector cells. One possible explanation is that temperature and/or CO_2 levels affect *atxA* expression, which in turn leads to toxin production. A later publication reported that temperature affected production of the transactivator protein but CO_2 levels did not (Dai and Koehler, 1997; Dai et al., 1995; Uchida et al., 1993).

The precise mechanisms by which CO_2 and *atxA* regulate expression of toxin genes remain unclear.

2.4. Treatment and Prevention

In the absence of previous immunity, resolution of anthrax requires antibiotic treatment prior to the onset of fulminant disease (Jernigan et al., 2001). Treatment is typically initiated with oral antibiotics, although for pulmonary anthrax or systemic dissemination, intravenous administration of antibiotics is recommended. Limited human data obtained from the U.S. anthrax patients in 2001 suggests that early detection and swift application of antibiotics can reliably prevent mortality and progression of the disease (Jernigan et al., 2001).

In August 2000 the Food and Drug Administration (FDA) approved the use of ciprofloxacin for treatment of postexposure anthrax (Meyerhoff et al., 2004). The CDC recommends ciprofloxacin or doxycycline in combination with at least one other antibiotic such as rifampin, vancomycin, or chloramphenicol for proper treatment (CDC, 2001a). Penicillin-resistant strains are emerging, and susceptibility to antimicrobials should be verified before deciding the course of treatment (Lalitha and Thomas, 1997). A 30 to 60-day course of antibiotics is the standard treatment. Data obtained from monkey models of inhalation anthrax indicate that spores can take as long as 100 days to germinate; therefore, close clinical observation should follow completion of antibiotic treatment (CDC, 2000, 2001a, b; Henderson et al., 1956). Alternative options include an extended 100-day regimen or postexposure vaccination in combination with antibiotic treatment (CDC, 2000; Friedlander et al., 1993b).

Although in the 2001 outbreak patients treated with antibiotics in early stages of the infection survived, Brookmeyer et. al. (2004) speculate that swift antibiotic treatment alone will be at best 70–80% effective in larger outbreak scenarios. Therefore, preexposure immunization may be necessary to achieve higher survival rates in the event of a large-scale bioterrorist attack. The Advisory Committee on Immunization Practices (ACIP) has recommended making anthrax vaccine available to individuals who are at risk of exposure to aerosolized anthrax spores (CDC, 2000).

Studies by Marcus et al. (2004) indicate that postexposure vaccination as a sole treatment is unlikely to be beneficial. The mean time to death in guinea pigs is 2–4 days after infection while the time required to achieve protection following booster immunizations is 8 days. Also, monkeys vaccinated immediately following aerosol exposure were no better protected than naive controls, while animals that received the vaccine along with antibiotics were protected (Friedlander et al., 1993b). These data strongly suggests that vaccination alone in a postexposure scenario will not allow sufficient time for anti-PA antibody titers to develop, and treatment should be supplemented with antibiotics and/or passive immunotherapy to increase chances of survival.

Failure of antibiotic treatment in late-stage disease suggests that the infection has persisted long enough to raise toxin concentrations to lethal levels. Furthermore, it is unlikely that antibiotics combined with vaccination in patients with advanced disease will prevent death. Therefore, immediate neutralization of anthrax toxin may be the only method of preventing mortality associated with the late phase of pulmonary anthrax. Although the CDC has recognized the potential benefit of having preimmune sera on hand in the event of future outbreaks, it remains to be determined how effective this treatment could be in late-stage human disease (Enserink, 2002).

2.5. Vaccines and Immunity

Immunologic protection against anthrax infection is primarily determined by the level of anti-PA antibody present at the time of exposure. In the absence of significant anti-PA antibodies, pulmonary anthrax or systemic LeTx intoxication invariably leads to death. The crucial nature of anti-PA antibodies to host survival can be attributed at least in part to their ability to (1) prevent germination of extracellular spores; (2) neutralize toxin; and (3) enhance macrophage-mediated killing of engulfed spores (Welkos et al., 2001, 2002). Although the individual contributions of each of these events to survival of the host are not completely understood, collectively they afford significant protection against the most serious form of anthrax disease. Lasting host immunity is therefore dependent on the establishment of B-memory and B-effector cell populations capable of providing a continuous supply of anti-PA-specific plasma cells.

The only anthrax vaccine licensed for human use in the U.S. is Anthrax Vaccine Adsorbed (AVA). It was licensed by the FDA in 1970 and is currently provided only to military personnel, individuals at high risk of occupational exposure, and to those recently exposed or suspected to have been exposed to aerosolized spores. The vaccine is derived from the cell-free filtrate of a nonencapsulated strain of *B. anthracis*. Although AVA contains multiple bacteria and media-derived components, the primary immunogen is PA-adsorbed onto aluminum hydroxide adjuvant (Ivins et al., 1996). This vaccine is far from ideal because it requires multiple vaccine doses and it has been reported to induce adverse effects in some vaccinees (Belyakov et al., 2000; Kerrison et al., 2002)

Patients receiving AVA are administered three subcutaneous injections at 0, 2, and 4 weeks followed by three more injections at 6, 12, and 18 months. In addition to the 6 injections, annual boosters are recommended for as long as immunity is required. Since immunological memory to PA in the absence of anti-PA antibodies is not necessarily protective in animal models, annual boosters are recommended to prevent titers from falling below minimal protective levels, which could leave vaccinated individuals at risk of infection.

Intramuscular vaccination with AVA induces primarily a systemic immune response characterized by high serum IgG levels capable of efficiently neutralizing toxin in passive transfer experiments (Sawada-Hirai et al., 2004). Studies in rhesus macaques have demonstrated that animals vaccinated with AVA were protected against aerosol challenge with 93 lethal doses (LD50) of the highly virulent Ames strain of *B. anthracis* (Ivins et al., 1998). Challenge studies in nonhuman primates represent the most reliable data available regarding the efficacy of AVA in humans since there are no confirmed cases of AVA vaccinees being exposed to infectious spores.

AVA and an equivalent vaccine available in the U.K. are both prepared from the culture filtrate of nonencapsulated strains of *B. anthracis* according to procedures that have been in place for over 40 years (Puziss et al., 1963). Both vaccines are administered parenterally (intramuscular injection) and provide robust systemic immunity but limited mucosal or cell-mediated immunity. Since most microorganisms typically enter via mucosal surfaces, it is believed that local immunity may aid in preventing the initial establishment of infection and, in combination with systemic immunity, increase the minimal infectious dose required to cause disease. Advances in protein purification, adjuvants, immunology, and vaccinology have provided opportunities for researchers to design more effective vaccines that can be administered through nonparenteral (needle-free) routes and that potentially provide both mucosal and systemic immunity to pathogens.

Given the evidence that protection against anthrax correlates strongly with anti-PA antibody titers, researchers are currently exploring the effectiveness of purified PA protein in next-generation vaccines with increased safety and efficacy (Pitt et al., 2001; Reuveny et al., 2001). Besides the obvious benefit of vaccinating people with well-defined formulations, expression of PA from vectors other than *B. anthracis* is safer and easier to standardize. In anticipation of phase I clinical trials, researchers at the National Cancer Institute have demonstrated successful adsorption and desorption of recombinant PA (rPA) to FDA-approved adjuvants with little loss of biologic viability (Jendrek et al., 2003). Other researchers have characterized the immune response to intranasal immunization using rPA in combination with various experimental adjuvants (Boyaka et al., 2003). Intranasal immunization of mice using cholera toxin (CT) as adjuvant elicited mucosal and systemic IgG responses as well as high levels of type 2 cytokines. Other novel approaches to vaccination include the use of transcutaneous immunization using a patch-based vaccine with *Escherichia coli* heat-labile toxin (LT) adjuvant (Kenney et al., 2004).

Continuing research into adjuvants, novel antigens, alternative routes of administration, and mechanisms of immune development will likely lead to improved anthrax vaccines for humans. Besides providing a benefit to overall public health, research on improved vaccines and therapeutics will provide the necessary tools to protect soldiers and civilians from the threat of biological warfare.

3. Plague

3.1. Introduction

Plague is a zoonotic infection caused by the Gram-negative coccobacillus *Y. pestis,* a Gram-negative rod that has a bipolar "safety pin" appearance on Giemsa or Wayson stains (Figure 3). Several rodent and lagomorph species are natural reservoirs for plague, and the disease is transmitted between animals by hematophagous fleas or by direct contact. Humans are accidental hosts and are commonly infected after the bite of an infected flea or via respiratory droplets from animals to humans or between humans. *Y. pestis* is believed to be responsible for the great epidemic of "Black Death" which killed millions of people in Europe in the Middle Ages. Infection with *Y. pestis* is no longer a preamble to death, since most infections are resolved after antibiotic treatment; however, cases of pneumonic plague that are not promptly diagnosed and treated can be fatal.

The causative agent of plague, *Y. pestis*, was discovered by French bacteriologist Alexandre Yersin in 1894. In 1898 Paul-Louis Simon observed that fleas were the natural vector of transmission to animals (Mollaret, 1999). Plague is one of the oldest recorded infectious diseases, and a recent publication points

FIGURE 3. Wayson stain of *Yersinia pestis* showing the characteristic "safety pin" appearance of the bacteria. (From CDC web page http://www.cdc.gov/ncidod/dvbid/plague/wayson.htm).

to its origin in ancient Egypt (Panagiotakopulu, 2004). Although many unconfirmed records of ancient plague epidemics abound in the literature, it is known that the first recorded pandemic or "Justinian" plague (AD 541–767) started in Egypt and spread to Europe and Africa. The second pandemic, known as the 'Black Death' (1346 to the early 1600s) spread from the Caspian Sea to Europe, and the third pandemic began in 1855 in the Yunnan region of China and spread globally via ships leaving from Hong Kong (Achtman et al., 1999; Perry and Fetherston, 1997).

Y. pestis can be found in more than 200 species of wild rodents present in most continents, and over 80 species of fleas are proven vectors of plague (Anisimov, 1999, 2002a, b; Brubaker, 1972, 1991; Perry and Fetherston, 1997).

Plague first entered the U.S. in 1899 when infected rats from a ship sailing from Hong Kong to San Francisco left the vessel and entered the city's sewer system. The San Francisco epidemic lasted 4 years and killed 118 people (Lipson, 1972). Plague in North America occurs in 15 western states of the U.S., in southwestern Canada on the border with the U.S., and in northern Mexico.

Although the improvement of living and sanitary standards worldwide has decreased the incidence of plague, the World Health Organization (WHO) reports that from 1954 to 1997 plague occurred in 38 countries, with 80,613 cases and 6,587 deaths (Dennis et al., 1999). The WHO recently reported that an outbreak of plague in Congo, Africa, started in October 2004 and caused over 20 deaths. In the U.S. bubonic plague occurs at a rate of 10–15 cases per year, mostly in veterinarians and other animal handlers (Chase, 2003; MMWR, 1996). It is clear from these epidemiological studies that this ancient scourge is far from being eradicated from the modern world.

Plague has been used as a biological weapon throughout history. Two of the most notable examples are Tartars in 1346, catapulting plague-infected corpses into Kaffa at the end of a 3-year siege, and the Japanese in the 1940s, breeding *Y. pestis*-infected fleas with the intention to release them from aircrafts over Chinese cities (Beeching et al., 2002; Christopher et al., 1997; Inglesby et al., 2000). The palpable threat of bioterrorism combined with the continuing occurrence of natural plague outbreaks emphasizes the need to understand the pathogenesis of this organism and to develop new prophylactic and therapeutic tools to control plague.

3.2. Pathogenesis

The genus *Yersinia* consists of 11 species, three of which, *Y. pestis*, *Y. enterocolitica*, and *Y. pseudotuberculosis*, are human pathogens. Evolutionary studies indicate that 20,000 years ago *Y. pestis* evolved from *Y. pseudotuberculosis*, and that both *Y. enterocolitica* and *Y. pseudotuberculosis* evolved from a common predecessor 1 million years earlier (Achtman et al., 1999; Wren, 2003). *Y. pestis* causes plague and is transmitted by insects or by droplets, whereas

Y. enterocolitica and *Y. pseudotuberculosis* are enteropathogens that are transmitted by food and water and enter the host through M cells in intestinal Peyer's patches (Autenrieth and Firsching, 1996; Clark and Jepson, 2003), followed by invasion of lymphoid tissues (Autenrieth and Schmidt, 2000; Wren, 2003) (Figure 4).

Depending on the mode of transmission and the immune status of the infected individual, *Y. pestis* can cause bubonic, septicemic, pneumonic, pharyngeal, cutaneous, or enteric plague. Bubonic, septicemic, and pneumonic plague are the most recognized forms and will be the only ones discussed here.

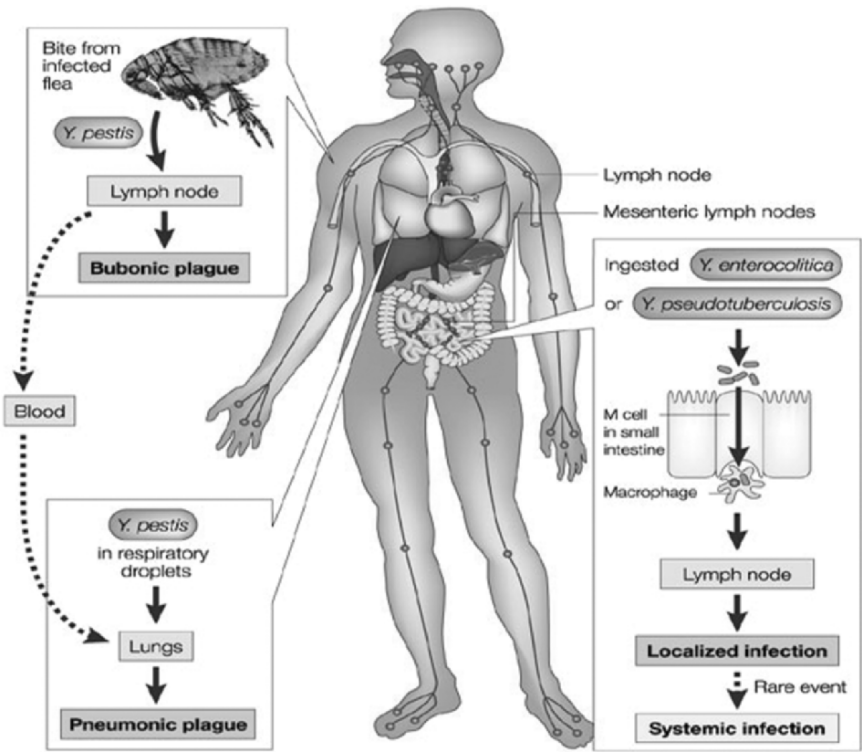

FIGURE 4. Steps in the transmission of pathogenic *Yersinia* in humans. *Yersinia pestis* has a rodent reservoir. Fleas infesting the rodents acquire *Y. pestis* from infected blood and transmit the bacterium to other rodents or occasionally to humans, causing bubonic plague. Pneumonic plague is transmitted from human to human through respiratory droplets or by artificially generated aerosols. In contrast, *Y. enterocolitica,* and *Y. pseudotuberculosis* are transmitted orally and enter the lymphatic system through the M cells of the small intestine. (Reproduced with permission from Wren, 2003.)

The bubonic plague is the most common form of the disease and occurs following a bite from a flea that has previously taken a blood meal from an infected animal (Brubaker, 2003). *Y. pestis* disseminates from the initial site of infection and drains to the local lymph nodes, especially axillary or inguinal, which rapidly become swollen, necrotic and painful. The swollen lymph nodes, known as "buboes", can get larger than a golf ball and constitute the classical pathognomonic feature of bubonic plague. Bacteremia often develops as bacteria from the bubo spill into the bloodstream, and large numbers of bacteria can be usually cultured from the blood (Butler, 1989; Crook and Tempest, 1992).

Septicemic plague arises when bacteremia occurs without notable bubo formation. It is characterized by fever, headache, malaise, and gastrointestinal disturbances. These symptoms are nonspecific, making the initial diagnosis difficult and delaying the start of antibiotic therapy. Even with medical treatment, about 30–50% of the cases are fatal (Hull et al., 1987).

Pneumonic plague, the most lethal form, arises after inhalation of bacteria-containing droplets, followed by colonization of the alveoli in the lungs with *Y. pestis*. The incubation period is 1–3 days, and patients with this type of infection produce bloody sputum that is highly contagious. Plague acquired this way has a 100% mortality rate if antibiotic treatment is not administered at the first signs of illness. *Y. pestis* pneumonia can also develop after initial bubonic or septicemic plague. Regardless of how it is acquired, pneumonic plague can spread rapidly within a population (Perry and Fetherston, 1997). This contagious and deadly form of plague would be the most likely outcome of an intentional bioterrorism attack using aerosolized bacteria.

One of the most interesting aspects of the pathogenesis of *Y. pestis* is the strategy used by the bacterium to ensure propagation from the insect vector. When a flea ingests blood containing *Y. pestis*, the bacteria start multiplying rapidly in the midgut of the insect. Two days after ingestion of infected blood, the stomach of the fleas have large masses containing fibrin, hemin, and bacteria, which continue to grow over a period of about 1 week. This produces a "blockage" that prevents the flea from ingesting food. To avoid starvation the insect needs to get rid of the blocking material by regurgitating the *Y. pestis*-laden mass. As the flea takes its next meal, it delivers the bacteria onto the wounded skin of the host (Gage and Kosoy, 2004).

3.3. Virulence

The most significant differences between *Y. pestis* and the other *Yersinia* species evolved after *Y. pestis* gained several genes by lateral transfer, and in the process acquired the ability to be transmitted by insect vectors and to disseminate through lymph and interstitial spaces. It is known that *Y. pestis* carries two plasmids not present in the enteropathogenic *Yersiniae*: pPla and pMT1 (Ferber and Brubaker, 1981). Additionally, some of the genes that facilitate invasion of enteropathogenic *Yersiniae* to the small intestine are

inactive in *Y. pestis* (*YadA, Inv*) (Brubaker, 2003). It has been postulated that the loss of these genes may contribute to the severity of plague (Perry and Fetherston, 1997).

Despite the genetic similarities with *Y. pseudotuberculosis*, *Y. pestis* causes a very different and much more severe disease (Parkhill et al., 2001). All pathogenic *Yersinia* species express virulence factors that enable them to enter, colonize, and multiply in lymphatic tissues and organs. Among these virulence factors are iron-binding siderophores, toxins, adhesins, and invasion molecules. Most importantly, all three pathogenic *Yersinia* species use a type III secretion system to inject effector proteins (Yops) into the host cell.

The pathogenicity of *Y. pestis* results from its ability to overcome the defenses of the host and to multiply rapidly, reaching high numbers in infected tissues and blood. *Y. pestis* is a facultative intracellular pathogen that is thought to be intracellular only during the early stages of infection, followed by extracellular growth at later stages (Brubaker, 1991; Straley et al., 1993). Therefore, to be a successful pathogen, *Y. pestis* needs effective mechanisms to survive both in the intracellular and extracellular compartments. Here, we present a summary of the most important virulence factors described in the plague bacillus.

3.3.1. Yersinia Outer-membrane Proteins

All pathogenic *Yersinia* species contain the 70 kb virulence plasmid pYV, also called low-calcium response plasmid (pLcr), which enables the bacteria to survive and multiply in the lymphoid tissue of their host (Brubaker, 1983; Cornelis et al., 1998). Curing of this plasmid results in total avirulence of the *Yersinia* strains studied thus far (Brubaker, 1983; Kutyrev et al., 1989). This plasmid encodes the Yop virulon, a system consisting of secreted effector proteins called Yersinia outer-membrane proteins (Yops) and a type III secretion apparatus that serves to inject Yops into the host cell. The injected Yops disturb the dynamics of the cytoskeleton and block the production of proinflammatory cytokines, thereby favoring the survival of the invading *Yersinia* (Bliska and Black, 1995; Fallman et al., 1995; Grosdent et al., 2002; Rosqvist et al., 1990). Of particular interest, YopH has a direct effect on inflammation by its ability to downregulate expression of costimulatory molecule B7.2, thereby modulating T cell- and B cell-mediated responses and possibly repressing monocyte chemotactic protein 1 (MCP-1).

3.3.2. V-antigen

The V-antigen, or LcrV protein, which is also encoded by the pYV plasmid, was assumed to be a protective antigen shortly after its discovery in 1956 by T. W. Burrows (Burrows, 1956; Burrows and Bacon, 1958; Une and Brubaker, 1984). In 1994, Motin demonstrated passive immunity mediated by antibodies against a staphylococcal A-LcrV fusion (PAV) (Motin et al., 1994). He also showed that injection of PAV prevented upregulation of IFN-γ and TNF-α by

LcrV-deficient mutants, providing additional evidence that this protein blocks the inflammatory response (Nakajima et al., 1995). It has since been shown that V-antigen serves two broad functions. First, V antigen (along with YopB and YopD) is responsible for selective targeting of effector proteins into the cytosol of infected cells by the type III secretion system (Cornelis, 2000; Cornelis et al., 1998). Second, V antigen is secreted into the environment (Fields et al., 1999), and can act on macrophages to induce the immunoregulatory cytokine IL-10 (Sing et al., 2002a). While the exact relevance of V antigen on virulence and immune evasion is still unclear, recent evidence points to V-antigen signaling through CD14 and TLR2 (Sing et al., 2002b).

3.3.3. F1 Capsule

The 96 kb pMT1 (also known as pFra) plasmid encodes the *Yersinia* murine toxin Ymt and the F1, or Caf1, capsule. The murine toxin has been shown to facilitate colonization of the flea and has phospholipase D activity that is toxic to mice (Hinnebusch et al., 2000; Rudolph et al., 1999). F1 is the major protein component of the capsule (Baker et al., 1952; Burrows, 1963) and lends resistance to *Y. pestis* against phagocytosis by macrophages (Cavanaugh and Randall, 1959; Du et al., 2002). Prolonged growth at 37˚C is necessary for capsular development (Du et al., 2002). Not surprisingly, *Y. pestis* does not synthesize F1 in the flea vector (Cavanaugh and Randall, 1959). Mutants unable to synthesize F1 occur in nature, but in general are not highly attenuated; probably because their ability to secrete type III Yops is enhanced (Davis et al., 1996; Drozdov et al., 1995; Du et al., 1995; Welkos et al., 1995). It has been reported that *Y. pestis* F1-negative strains are virulent in mice (Friedlander et al., 1995), African green monkeys (Davis et al., 1996), and humans (Winter et al., 1960).

3.3.4. Plasminogen Activator Pla

The 9.6 kb pPla, or pPst, encodes the plasminogen activator Pla, a coagulase/fibrinolysin that interferes with blood coagulation and complement pathways (Beesley et al., 1967; Sodeinde and Goguen, 1988). Pla is very important for the dissemination of *Y. pestis* from peripheral infections; Pla mutants injected subcutaneously produced only a localized infection with high numbers of inflammatory cells in lesions when compared with nonmutated strains (Sodeinde et al., 1992; Welkos et al., 1997). It is believed that during the emergence of *Y. pestis* from *Y. pseudotuberculosis*, fibrillar adhesin YadA and cell invasin Inv were replaced by tissue invasin Pla (Finlay and Falkow, 1997; Perry and Fetherston, 1997).

In summary, the Pla protease allows *Y. pestis* to be highly invasive, the capsule offers immediate resistance to phagocytosis by macrophages, Yop injection cripples the immune response of surrounding cells, and secreted LcrV causes a systemic anti-inflammatory response by reducing IFN-γ and TNF-α and increasing IL-10 production.

3.4. Treatment

The key to treating plague, particularly the pneumonic or septicemic form, is rapid diagnosis and administration of prompt antibiotic therapy. Most therapeutic guidelines suggest using streptomycin or gentamicin as first-line therapy against plague, with ciprofloxacin as optional treatment. Additionally, patients should be monitored for septic shock in an intensive care unit. Persons who come into contact with plague patients should receive antibiotic prophylaxis for 7 days. In order to prevent human-to-human transmission, quarantine should be observed until the patient has received at least 4 days of antibiotic treatment. For the other clinical manifestations of the disease, e.g., bubonic plague, patients should be isolated for the first 48 h after the initiation of treatment (Bossi et al., 2004).

3.5. Vaccines and Immunity

The development of an effective vaccine against plague is desirable since plague is endemic in many parts of the world and there is an ever-present likelihood of the illegitimate use of *Y. pestis* by bioterrorists. An ideal vaccine should be safe, require few doses, cause little or no side effects, be easy to administer, and be inexpensive to manufacture in mass quantities. More specifically, an ideal vaccine would induce sufficient levels of effector memory cells capable of rapid expansion upon exposure to plague. A number of candidate vaccines have been described and tested, among which the most recently described subunit vaccines have great potential for successful development into a practical vaccine for human use.

The Cutter USP, or killed whole cell (KWC), vaccine is a formaldehyde KWC preparation developed from a virulent *Y. pestis* strain. This preparation is expensive, has a high degree of heterogeneity, has variable endotoxin content, requires frequent boosters, and can cause a variety of local and systemic side effects (Russell et al., 1995). More importantly, there have been cases of pneumonic plague reported in vaccinated individuals indicating inadequate immunity at mucosal surfaces (Meyer, 1970). The Cutter vaccine is no longer approved for use in the U.S.

Live attenuated EV76 vaccine, which has been used since 1908, is a nonpigmented mutant of *Y. pestis* that is unable to assimilate chromogenic substances such as Congo Red (Meyer, 1970). The live EV76 vaccine induces better protection against *Y. pestis* challenge than the KWC vaccine, but the live vaccine strain is not fully avirulent and can cause disease in the mouse model (Russell et al., 1995). In humans, the EV76 vaccine has been tested extensively but its effectiveness is questionable (Meyer, 1970; Meyer et al., 1974). Furthermore, significant variability in efficacy and side effects is seen between various EV76 preparations (Russell et al., 1995).

While the EV76 vaccine is not safely attenuated for use in humans, it does yield protection against both bubonic and pneumonic plague, suggesting that

attenuated bacteria may be a possible venue for a safe and effective plague vaccine. Other attenuated bacterial species carrying plasmids coding for plague antigens can elicit significant systemic and mucosal anti-*Yersinia* immune responses. Studies have shown increased protection against plague infection following subcutaneous immunization of mice with attenuated *Salmonella typhimurium* expressing F1 protein (Oyston et al., 1995; Titball et al., 1997). However, mice and African green monkeys immunized this way are not protected against all *Y. pestis* strains (Friedlander et al., 1995).

Other studies showed that an oral vaccine with attenuated *S. typhimurium* expressing V antigen protected mice from subcutaneous plague challenge (Garmory et al., 2003). Immunization studies with *S. typhimurium* expressing both F1 and V antigen induced 1,000 times greater protection than that induced by *S. typhimurium* expressing F1 alone (Leary et al., 1997).

The use of plasmid DNA encoding protective antigens has been demonstrated to be an effective method of generating immune responses against several viral and bacterial pathogens. A DNA construct encoding the V antigen was shown to induce immune responses in the mouse after delivery of the plasmid either intramuscularly or intradermally with a gene gun (Bennett et al., 1999; Garmory et al., 2004). It has also been determined that the inclusion of other antigens (*B. anthracis* PA) in the first DNA immunization and in the subsequent protein boost enhanced the protective immune response to *Y. pestis* (Williamson et al., 2002). Significant antibody responses were also observed following a 3 or 4 immunization regimen with optimized DNA vaccines encoding the F1 protein (Grosfeld et al., 2003).

Despite the potential reported from the vaccine strategies listed above, there are many known disadvantages to killed, live attenuated, and DNA vaccines, including negative side effects, lack of protection against aerosolized plague, and the need for frequent boosters. To overcome these disadvantages and to improve efficacy, numerous studies are now being conducted with acellular vaccines made up of recombinant plague proteins (Health et al., 1998; Williamson et al., 1997). Many surface-expressed plague proteins have been studied and have been shown to induce high levels of specific antibody (Andrews et al., 1999; Benner et al., 1999). However, only immune responses against F1 and V antigen proteins provided protection against *Y. pestis* challenge (Titball and Williamson, 2001). F1 and V antigen have been expressed as recombinant proteins (Leary et al., 1995; Miller et al., 1998) and have been shown to induce protective responses when used separately (Anderson et al., 1996; Leary et al., 1995; Roggenkamp et al., 1997).

A more recent approach to vaccination is the use of a genetic F1–V fusion (Heath et al., 1998), coadministered with alum. This vaccine induces significant protection in several animal models (Anderson et al., 1998; Heath et al., 1998; Jones et al., 2000, 2003; Williamson et al., 1995, 1996, 1997, 2000). A study in mice, evaluating the effect of using different routes of vaccination for the initial priming and the subsequent boosting, indicated that long-lasting high titer

specific antibody levels were developed whether mice were boosted by the same or different route of administration (Glynn et al., 2005).

Another novel approach to antiplague vaccination is the microencapsulation of antigenic peptides. This provides an adjuvant effect by improving uptake into antigen presenting cells (APCs) (Morris et al., 1994) and sustained release of the antigen over long periods of time (Eldridge et al., 1991; Nakaoka et al., 1996). Several studies have demonstrated successful inclusion of F1 and V antigen into microspheres (Eyles et al., 1998a, b; Spiers et al., 1999; Williamson et al., 1996). Immunization with these microcapsules leads to increased protection, which is further enhanced by inclusion of a mucosal adjuvant (Eyles et al., 1998a; Williamson et al., 1996).

Many studies have indicated that the strongest correlate of protection is the presence of antibodies against *Y. pestis* antigens (Elvin and Williamson, 2000; Williamson et al., 1999). Circulating anti-F1 and anti-V antibody presumably functions to bind surface-exposed or secreted *Y. pestis* proteins. It is known that innate immune responses are necessary to resolve the necrotic lesion(s) caused by a natural *Y. pestis* infection (Brubaker, 2003). However, upon *Y. pestis* infection, the V antigen inhibits innate immunity by inducing upregulation of IL-10 and downregulation of IFN-γ and TNF-α, thereby preventing granuloma formation and instead allowing the development of necrotic lesions (Moore et al., 2001; Wang et al., 1995). This dramatic decrease of Th1 cytokines is shown to be very characteristic of plague. Studies have shown that passive administration of anti-V serum prevents the downregulation of IFN-γ and TNF-α (Nakajima and Brubaker, 1993; Nakajima et al., 1995). A vaccine that generates a type 2 antibody response against V antigen could conceivably protect against disease by neutralizing the physical and chemical effects of the V antigen on innate immunity. A positive correlation between decreasing levels of antibody titers, especially IgG1, and decreasing protection against challenge has been established (Williamson et al., 1999).

Recent studies, however, suggest greater involvement of a type 1 response in protection against plague. Delivery of F1 and V with Ribi adjuvant to IL-4 receptor knockout mice induced a predominantly type 1 response. Yet, transfer of serum from these animals into B cell knockout mice was sufficient to protect the passively transferred animals from challenge (Elvin and Williamson, 2000). Elvin and Williamson (2004) observed that F1- and V-immunized Stat 4 knockout mice (with a defect in the type 1 cytokines IFN-γ and IL-12) do not survive *Y. pestis* challenge, whereas immunized Stat 6 knockout mice (with a defect in type 2 cytokines IL-4 and IL-13) do not show compromised protection compared to immunized normal syngeneic mice.

The involvement of both type 1 and 2 immune responses in protection against plague is a practical finding in the sense that Th1/Th2 protective responses rarely, if ever, exist separately in humans. These new studies illuminate the importance of examining both types of immune responses in experimental immune response and challenge models.

4. Tularemia

4.1. Introduction

Tularemia is a severe and potentially lethal zoonotic disease in humans caused by the Gram-negative bacterium *Francisella tularensis*. It is considered a dangerous potential biological weapon because it is highly infectious and can cause serious illness and death, especially after inhalation of the aerosolized bacteria.

Tularemia was first recognized in Japan in 1837. In 1911, American investigators described a plague-like illness killing rodents in Tulare County, California. The isolated causative organism was named *Bacterium tularense*. In 1921, Edward Francis linked the etiological agent as being the cause of multiple tularemia syndromes in animals and humans. In honor of Francis, who devoted his life to researching the pathogenesis of this organism, the bacterium was later renamed *F. tularensis* (Dennis et al., 2001).

F. tularensis has been recognized as a biothreat for decades. Between 1932 and 1945, the Japanese studied tularemia in their biowarfare research facilities in Manchuria (see Historical Landmarks). It has been suggested that the outbreaks of tularemia in Russian and German soldiers during World War II might have been intentionally caused. In the 1950s and 1960s, there were programs in the U.S. and in the former U.S.S.R. to develop tularemia as a biological weapon, and the organism was grown and stockpiled by both countries. After the U.S. signed the Biological and Toxic Weapons Convention in 1972, all the stockpiled biological weapons were destroyed (Dennis et al., 2001).

Tularemia is a disease that occurs naturally in many parts of the world and is particularly prevalent in the northern hemisphere. In the U.S., the number of cases declined from several thousand in the 1930s to less than 200 in 2000. Natural reservoirs of *F. tularensis* include multiple species of animals such as rodents, rabbits, hares, voles, and water rats; but the bacteria can also be found in insects and protozoans, and water transmission of the disease from wild animals to humans can occur via an arthropod vector. Several species of ticks (*Dermacentor reticulatus*, *Ixodes ricinus*), mosquitoes (*Anopheles*, *Aedes*), and biting flies have been implicated in transmission (Ellis et al., 2002; Oyston et al., 2004). Direct transmission from person to person has not been documented.

F. tularensis is a nonmotile, encapsulated, nonspore-forming, fastidious, Gram-negative coccobacillus. Four distinct subspecies of *F. tularensis* have been described based on virulence, O-antigen structure, biochemical tests, and geographical location where the organism is found. The subspecies are *tularensis* (type A), *holartica* (type B), *novicida*, and *mediasiatica*. Of these, the subspecies *tularensis* is the most virulent, with an infectious dose of less than 10 CFU and a mortality rate of 5–30% in the absence of treatment. Subspecies *holartica* and *mediasiatica* have low virulence, and subspecies *novicida* is only reported to cause disease in immunocompromised humans (Ellis et al., 2002; Titball et al., 2003).

4.2. Pathogenesis

F. tularensis is a zoonotic disease found mostly in rodents and lagomorphs. Humans are incidental hosts that can become infected by different routes of exposure. Infection might occur after: the bite from an infected insect, handling infected animal carcasses, drinking contaminated water, eating contaminated meat, or inhaling infectious aerosolized droplets. The route and site of transmission, the virulence of the infecting organism, and the immune status of the host determine the clinical presentation of the disease. The incidence of tularemia is higher in people with an occupational risk, such as hunters, butchers, farmers, and laboratory workers (Dennis et al., 2001; Feldman et al., 2001; Lawler, 2005). The six clinical types of tularemia described have significant symptomatology overlap between them (Lamps et al., 2004). The clinical presentations are: ulceroglandular, glandular, oculoglandular, oropharyngeal, pneumonic, typhoidal, and septic tularemia. The pneumonic form is the most severe and has the highest rate of mortality, but most clinical types can include pulmonary involvement.

The most common type is ulceroglandular tularemia, which appears after an arthropod bite, or after handling contaminated material. An inflamed local ulcer develops at the site of infection, which later becomes a pustule. Although the ulcer heals, bacteria can disseminate to the local lymph nodes, which become grossly swollen and tender. These enlarged lymph nodes closely resemble the characteristic "buboes" seen in bubonic plague.

Oculoglandular tularemia occurs after accidental inoculation of the conjunctiva with infected fingers. The disease is manifested by inflammation and the appearance of ulcers in the eye. Glandular tularemia is characterized by lymphadenopathy in the absence of an ulcer.

Oropharyngeal or gastrointestinal tularemia usually develops after the ingestion of contaminated water or food. Infected patients develop pharyngitis, tonsillitis, and cervical lymphadenopathy. Typhoidal and septic tularemia are similar clinical syndromes. They are severe, potentially lethal infections and are manifested after systemic dissemination of the organism, without evident cutaneous or mucosal involvement.

Respiratory tularemia, the most serious form of the disease, occurs after inhalation of infected aerosols or after hematogenous dissemination from a localized infection. This produces a severe, febrile illness, with initial flu-like symptoms progressing to bronchiolitis, pleuropneumonitis, and hilar lymphadenitis, with many patients developing acute symptoms 3–5 days after exposure. Pulmonary tularemia can progress rapidly and lead to acute respiratory failure and death (Oyston et al., 2004; Titball et al., 2003).

4.3. Virulence

The virulence factors that enable *F. tularensis* to be such a successful pathogen have not yet been identified. *In vivo*, *F. tularensis* is a facultative intracellular parasite with a predilection for macrophages (Figure 5). The

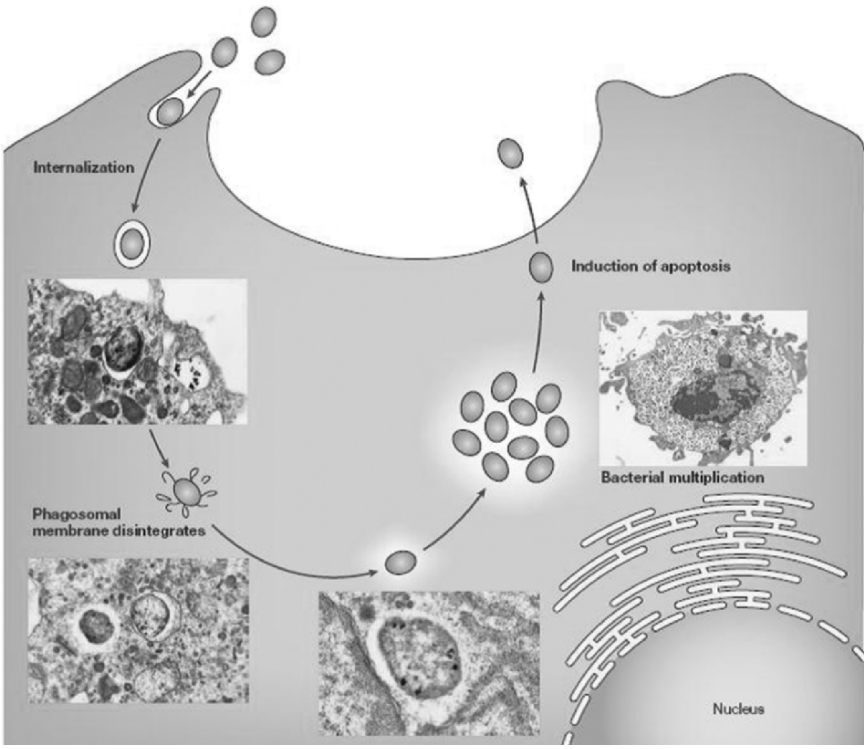

FIGURE 5. The intracellular lifestyle of *Francisella tularensis*. Steps in the intracellular replication cycle of *F. tularensis* resemble those observed for replication of *Listeria monocytogenes* inside macrophages. (Reproduced with permission from Oyston et al., 2004.)

bacteria enter macrophages without activating the respiratory burst, disrupts the phagosome membrane, and gains access to the macrophagic cytoplasm where rapid multiplication takes place. A bacterial acid phosphatase encoded by AcpA has been shown to inhibit the respiratory burst (Reilly et al., 1996). However, the role of this protein in virulence is tentative at best, since mutant Acp strains of *F. tularensis* subspecies *novicida* are still virulent in mice and can replicate in macrophages (Baron et al., 1999).

Proteomic studies have revealed that four *F. tularensis* proteins ranging in size from 20 to 70 kDa are upregulated when the bacteria are grown inside macrophages. The identity of these proteins and the role they play in virulence have not yet been elucidated (Golovliov et al., 1997). It is known that a 23 kDa protein encoded by IglC plays a role in intracellular multiplication of the subspecies *holartica* and *novicida*. Other genes such as MinD (Anthony et al., 1994) and those controlled by the operon mglAB (Lauriano et al., 2004) have been implicated in pathogenicity.

The LPS of *F. tularensis* is different from other Gram-negative bacteria LPS, as it does not seem to have a role as a typical proinflammatory endotoxin. *Francisella* LPS displays phase variation *in vitro*, producing phenotypically distinct colonies. However, the significance of this LPS change to virulence and to human disease is not known (Cowley et al., 1996). Also, it is known that the capsule is essential for serum resistance, but not necessary for survival after phagocytosis by polymorphonuclear leukocytes (Sandstrom et al., 1988).

The recently published complete genomic sequence of *F. tularensis* strain SchuS4 (the subspecies *tularensis* type strain) constitutes a new powerful tool for the identification and characterization of virulence factors in *Francisella*. The sequence revealed previously uncharacterized genes encoding type IV pili, a surface polysaccharide and iron-acquisition systems, as well as several virulence-associated genes located in a putative pathogenicity island (Larsson et al., 2005).

4.4. Vaccines and Immunity

A live vaccine strain (LVS) derived from attenuated *F. tularensis* has been used since 1960 to protect at-risk individuals. This vaccine has been used in the U.S. and Europe as an investigational new drug (IND), but it has never been fully licensed. LVS vaccine, administered by scarification, was shown to reduce the incidence of aerosolized tularemia from 5.7 to 0.27 cases per 1,000 at-risk workers, although it did not reduce the frequency of ulceroglandular disease (Burke, 1977). Further studies in human volunteers indicated that vaccination with LVS did not afford complete protection against a high-dose *F. tularensis* aerosol challenge (Eigelsbach et al., 1967). Other routes of administration of LVS (such as oral and nasal) have been shown to induce equal or better levels of protection against aerosolized *F. tularensis* challenge.

Although strong evidence indicates that LVS induces significant levels of protection, the FDA withdrew the IND status of LVS and is currently reviewing its use. Because the nature of the attenuating mutations in LVS is not known, there are legitimate concerns about the possibility of reverting to full virulence, or about the effects of this live vaccine on immunocompromised individuals.

In search for a better tularemia vaccine, other approaches have been investigated. Killed whole cell *F. tularensis* vaccines are protective only against low systemic challenges in animals, while in humans this vaccine reduced the number of infections and altered the course of infection (Ellis et al., 2002). The development of purified subunit vaccines has been hampered by our limited knowledge of the virulence factors and/or the protective immunogens of this organism. Vaccination of mice with LPS obtained from LVS induces varying degrees of protection against aerogenic or systemic challenge (Conlan et al., 2002b, 2003). Several outer-membrane proteins have been tested as potential vaccines against tularemia, but so far, no definite protective antigens have been identified.

An important piece of the puzzle for rational vaccine design is the understanding of the necessary immune responses to evoke protection. The immune correlates of protection against tularemia have not yet been identified. It has been reported that CD4+ and CD8+ cells (Conlan et al., 1994) as well as IFN-γ and IL-12 are necessary for protection (Anthony et al., 1989; Conlan et al., 2002a; Duckett et al., 2005; Elkins et al., 2002), but the exact contribution of these responses to clinical protection is not known.

The recently published genome sequence of *F. tularensis* strain SchuS4 (Larsson et al., 2005) is likely to provide new insights into the nature of previously uncharacterized protective antigens or might reveal new targets for immuno intervention or treatment against tularemia.

Questions to Consider

1. What would be the ideal vaccination approach against bacterial biowarfare agents?

An ideal vaccine should be safe, require few doses, induce long-lasting and rapidly developed protection, cause little or no side effects, be easy to administer, and be inexpensive to manufacture. In addition, this ideal vaccine should prevent the infection instead of resolving an established infection. For example, an ideal vaccine against pulmonary anthrax would prevent the germination of spores within the alveolar macrophages, thereby eliminating the chances of vegetative bacilli ever reaching internal organs.

2. What is the advantage for the anthrax bacilli to make a toxin that can bind to a receptor present on a wide variety of eukaryotic cells?

A recurring theme on the pathogenicity of many bacterial toxins is the subversion of ubiquitous host membrane molecules as specific receptors for their own toxin-binding subunits. In the case of the two identified anthrax toxin receptors, TEM8 is a membrane protein implicated in the neovascularization of human colon tumors and developing mouse embryos, while CMG2 is expressed in endothelial cells during capillary morphogenesis. However, the exact role of these proteins in normal cellular function and the reason why anthrax PA binds to them with such high affinity are not yet exactly known.

3. Theoretically speaking, which bacterial pathogen would be the most efficient "weapon of mass infection"?

The dissemination of anthrax via the U.S. postal system is an example of how even very small quantities of a biological pathogen spread intentionally can create a state of panic, produce great disruption to society and result in huge economic losses. Although many of the potential biothreats have this capacity, the most feared biowarfare agents are those that are highly infectious and can disseminate from person to person. Among the three category A bacterial pathogens discussed here, *Y. pestis* is the only one that can be spread by aerosol droplets between humans.

4. Considering that several bacterial biowarfare agents are easily obtainable from nature, why do you think there have not been many instances of successful mass bioterrorism using these agents?

The manufacture of lethal biological aerosols is, for the most part, beyond the capacity of individuals or groups without access to advanced biotechnology. The "weaponization" of microorganisms requires the concerted effort of professionally trained microbiologists, and industrial and manufacturing engineers. The process would need to be carried out in highly sophisticated laboratories to ensure that the aerosolizable microbial particles have the physical characteristics (e.g., density, weight, size), are viable, highly infectious, and virulent, and are properly contained, packed, and distributed.

5. Could anthrax toxin have beneficial uses for human health?

The use of various bacterial toxins as weapons to kill specific populations of cells (e.g., tumor cells) has been amply investigated using other potent bacterial toxins (i.e., diphtheria toxin and botulinum toxin). Although the potential therapeutic uses of anthrax toxin have not been investigated, it is conceivable to imagine that the toxin could be genetically modified to target specific cells for therapeutic purposes.

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